Diminished lung function, viral infections and chronic respiratory morbidity in prematurely born infants

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Abstract

Aims To assess the impact of RSV and other viral lower respiratory tract infections (LRTIs) on chronic respiratory morbidity in prematurely born infants and to investigate whether there were any functional or genetic predisposing factors.

Methods One hundred and fifty three prematurely born infants were followed until one year corrected age with approximately half followed until two years of age. Lung function was measured at 36 weeks postmenstrual age (PMA) and one year corrected age. Blood or buccal swabs were taken for single nucleotide polymorphism (SNP) analysis. Following neonatal unit discharge, a nasopharyngeal aspirate (NPA) was taken whenever an infant had a LRTI. NPAs were analysed by real time PCR for 13 viruses. At one and two years corrected age healthcare utilisation and costs of care were calculated and parents completed a respiratory health related questionnaire and a diary card for one month.

Results Infants developing RSV or other viral LRTIs requiring hospitalisation had reduced premorbid lung function compared to infants not hospitalised. Infants developing rhinovirus LRTIs had increased healthcare utilisation, cost of care and wheeze at one year corrected age. Infants developing RSV LRTIs had reduced lung function at one year corrected age. Prematurity was found to be a risk factor for developing RSV or other viral LRTIs but not influenza A (H1N1) LRTIs. A SNP in ADAM33 was associated with an increased risk of developing RSV LRTIs, but not with significant differences in 36 week PMA lung function results. SNPs in several genes were associated with increased chronic respiratory morbidity (IL10, NOS2A, SFTPC, MMP16 and VDR) and reduced lung function at one year (MMP16, NOS2A, SFTPC and VDR) in infants who had had RSV LRTIs.

Conclusion In prematurely born infants, RSV and other viral LRTIs were associated with increased chronic respiratory morbidity at follow up, with some infants being

genetically predisposed to this after RSV LRTI. Premorbid abnormal lung function predisposed to severe RSV and a SNP in the ADAM33 gene predisposed to RSV LRTIs.

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Publications arising from this thesis

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2. Drysdale SB, Alcazar M, Wilson T, Smith M, Zuckerman M, Wedderburn CJ, Broughton S, Rafferty GF, Johnston SL and Greenough A. Pandemic influenza A (H1N1) virus 2009 in a prospectively followed cohort of prematurely born infants. *Pediatr Infect Dis J* (2012); 31(1):91-92.
3. Drysdale SB, Alcazar-Paris M, Wilson T, Smith M, Zuckerman M, Broughton S, Rafferty GF, Peacock JL, Johnston SL and Greenough A. Rhinovirus infection and healthcare utilisation in prematurely born infants. *Eur Respir J* (2013); 42:1029-1036
4. Drysdale SB, Alcazar M, Wilson T, Smith M, Zuckerman M, Lauinger IL, Tong CYW, Broughton S, Rafferty GF, Johnston SL and Greenough A. Respiratory outcome of prematurely born infants following human rhinovirus A and C infections. *Eur J Pediatr* (2014) 173:913–919.
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List of abbreviations

A and E	Accident and Emergency
ADAM 33	A disintegrin and metalloprotease 33
AdV	Adenovirus
ATPS	Ambient temperature and pressure, saturated
ATS	American thoracic society
AUC	Area under curve
bp	Base pairs
BPD	Bronchopulmonary disease
BNFC	British National Formulary for children
BTPS	Body temperature and pressure, saturated
cDNA	Complimentary DNA
CLD	Chronic lung disease
CPAP	Continuous Positive Airway Pressure
C_{rs}	Respiratory system compliance
Ct	Cycle threshold
DFA	Direct fluorescent-antigen
DNA	Deoxyribonucleic acid
EBS	Equilibrated Bio-systems
ERS	European respiratory society
EV	Enterovirus
FA	Influenza type A
FB	Influenza type B
FEF_{25-75}	Forced expiratory flow from 25-75% of vital capacity
FEF_{50}	Forced expiratory flow at 50% of vital capacity
FEF_{75}	Forced expiratory flow at 75% of vital capacity
$FEV_{0.75}$	Forced expiratory volume at 0.75 seconds
FEV1	Forced expiratory volume in one second
FRET	Förster resonance energy transfer
FRC	Functional residual capacity
FRC_{He}	FRC measured by helium gas dilution
FRC_{MBW}	FRC measured by the multiple breath wash-in/out method
FRC_{pleth}	FRC measured by plethysmography
GA	Gestational age
GP	General Practitioner
HA	Haemagglutanin
HBIR	Hering-Breuer inflation reflex
HBOV	Human bocavirus
HDU	High dependency unit

HMPV	Human metapneumovirus
HPA	Health Protection Agency
HRV	Human rhinovirus
IC	Internal control
ICU	Intensive care unit
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
KCH	King's College Hospital
KLRG1	Killer cell lectin-like receptor G1
LCI	Lung clearance index
LRTI	Lower respiratory tract infection
MBL	Mannose binding lectin
MBW	Multiple breath wash-in/out
MEF_{25-50}	Maximal expiratory flow from 25-50% of vital capacity
MEF_{50}	Maximal expiratory flow at 50% of vital capacity
MM	Molar mass
MMP	Matrix metalloproteinase
NFκB1A	Nuclear factor-κ-B 1A
NHS	National Health Service
NA	Neuraminidase
NICU	Neonatal intensive care unit
nm	Nanometre
NNT	Number needed to treat
NOS2A	Nitric oxide synthase type 2A
NPA	Nasopharyngeal aspirate
OR	Odds ratio
ONS	Office for National Statistics
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEFR	Peak expiratory flow rate
PEV	Parechovirus
PF	Parainfluenza virus
PICU	Paediatric intensive care unit
PMA	Postmenstrual age
R_{aw}	Airway resistance
RCT	Randomised controlled trial
RDS	Respiratory distress syndrome
RNA	Ribonucleic acid

RNase	RNA lytic enzyme
ROC	Receiver operating characteristic curve
R_{rs}	Respiratory system resistance
rs	reference SNP
RIE	Royal Infirmary of Edinburgh
RIVM	National Institute for Public Health and the Environment, The Netherlands
RSV	Respiratory syncytial virus
RSV-IGIV	RSV intravenous immunoglobulin
RTC	Rapid thoraco-abdominal compression
rt-PCR	Real-time PCR
RT-PCR	Reverse transcriptase PCR
RV	Residual Volume
SCBU	Special care baby unit
SD	Standard deviation
SF6	Sulphur hexafluoride
SFTPC	Pulmonary surfactant protein C
sG_{aw}	Specific airways conductance
SNP	Single nucleotide polymorphism
SOT	Single occlusion technique
TGFβR1	Transforming growth factor-β receptor-1
T_H1	Type 1 T helper cell
TLC	Total lung capacity
tPTEF	Peak tidal expiratory flow
tPTEF/tE	Time to peak tidal expiratory flow as a proportion of total expiratory time
Tr	Response time
Trs	Time constant
URTI	Upper respiratory tract infection
USFM	Ultrasonic flowmeter
UV	Ultra violet
VC	Vital capacity
VDR	Vitamin D receptor
VI	Ventilation inhomogeneity
V_{max} FRC	Maximal flow at FRC
V_{pleth}	Measured volume inside the plethysmograph
VZV	Varicella Zoster virus

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Chapter 1: Introduction

1.1 Introduction

Viruses frequently cause respiratory tract infections especially in children less than five years of age (Pitman et al. 2007). Respiratory syncytial virus (RSV) infects almost all children by two years of age (Glezen et al. 1986). Up to 80% of hospital admissions for bronchiolitis are due to RSV, and RSV LRTIs have been associated with chronic respiratory morbidity. Many other viruses, however, are also associated with respiratory illnesses in children including rhinovirus, influenza, parainfluenza, adenovirus, enterovirus and parechovirus, as well as viruses that have only more recently been identified, for example human metapneumovirus and human bocavirus. All these viruses can present with either a mild upper respiratory tract infection or a lower respiratory tract infection (LRTI). LRTIs are characterised by coryza, cough, wheeze and dyspnoea and can be mild (requiring no medical attention) to severe (requiring mechanical ventilation).

1.1 Respiratory viruses

Viruses are classified by the International Committee of Taxonomy of Viruses (ICTV) (ICTV 2009) (Table 1.1).

Table 1.1: International Committee on Taxonomy of Viruses classification of viruses

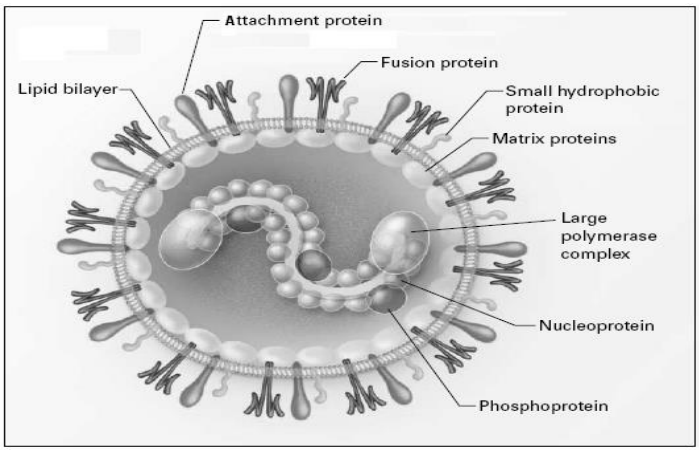
Order	Family	Subfamily	Genus	Species	Genome
Mononegavirales	*Paramyxoviridae*	*Paramyxovirinae*	*Respirovirus*	*Parainfluenza types 1 and 3*	Non-segmented, positive sense, single stranded RNA
			Rubulavirus	*Parainfluenza type 2*	Non-segmented, positive sense, single stranded RNA
			Pneumovirus	*RSV A* and *RSV B*	Non-segmented, negative sense, single stranded RNA
			Metapneumovirus	*Human metapneumovirus*	Non-segmented, positive sense, single stranded RNA
Picornavirales	*Picornaviridae*	No subfamily classified	*Enterovirus*	*Enterovirus*	Non-segmented, positive sense, single stranded RNA
				Rhinovirus	Non-segmented, positive sense, single stranded RNA
			Parechovirus	*Parechovirus*	Non-segmented, positive sense, single stranded RNA
No order assigned	*Orthomyxoviridae*	No subfamily classified	*Influenza A* and *B*	*Influenza types A and B*	Segmented, negative sense, single stranded RNA
	Adenoviridae	No subfamily classified	*Mastadenovirus*	*Adenovirus*	Non-segmented, double stranded DNA
	Parvoviridae	*Parvovirinae*	*Bocavirus*	*Human Bocavirus* is not classified	Non-segmented, negative sense, single stranded DNA

1.1.1 Respiratory Syncytial Virus

Respiratory Syncytial Virus (RSV) is classified by the ICTV as of the order Mononegavirales, family *Paramyxoviridae*, subfamily *Pneumovirinae*, and genus *Pneumovirus* (ICTV 2009) (Table 1.1). RSV is an enveloped, non-segmented virus with a negative sense, single-stranded RNA genome, containing ten genes encoding various proteins (Figure 1.1). The ten proteins can be divided into eight structural and two non-structural proteins. The structural proteins include the nucleoprotein (the major RNA binding nucleocapsid protein), the large polymerase complex and phosphoproteins (both involved in RNA dependent RNA polymerase activity), the fusion (F) protein (which is involved in cell penetration and protection), glycoprotein (G protein) (an attachment and protective protein), small hydrophobic (SH) protein and two matrix (M, M2) proteins (involved in attachment) (Hall 2001; Hacking & Hull 2002). The two non-structural proteins antagonise α and β interferon (Hacking & Hull 2002).

Figure 1.1: The structure of Respiratory Syncytial Virus

Adapted from (Hall 2001).



There are two distinct subgroups of RSV, RSV A and RSV B, defined by antigenic variation mainly in the G protein (Mufson et al. 1985). Both strains circulate simultaneously with the relative proportions of each strain varying year by year and by geographic location, although the majority of studies show RSV A is more often the dominant strain. Two large studies (Taylor et al. 1989; Hall et al. 1990) each including over 1000 infants reported similar results. Hall et al looked at the different strains over a 15 year period and demonstrated RSV A to be dominant (>80% of all RSV positive samples) in nine of the 15 years, RSV B to be dominant in four of the 15 years and similar proportions of the two strains in the remaining years (Hall et al. 1990). Taylor et al reported RSV A was dominant (>80% of isolates were RSV A) in six of the 15 years, whereas RSV B was borderline dominant in only one year (68% of isolates were RSV B), in the other years there was similar proportions of each subgroup (Taylor et al. 1989). RSV A dominance has been found in two more recently reported studies. RSV A was dominant (>50% of cases) in Belgium in seven of the ten years from 1996-2006 (Zlateva et al. 2007) and in Germany in seven of the nine years from 1998-2007 (Reiche & Schweiger 2009).

1.1.2 Rhinovirus

Rhinoviruses (HRV) are very small (approximately 27–30 nm in diameter) ribonucleic acid viruses consisting of a simple viral capsid and a single strand of RNA. The capsid contains four proteins, VP1–VP4, arranged in 60 repeating protomeric units in an icosahedron (Fendrick 2003). Rhinoviruses

consist of over 100 serotypes classified into three groups (A, B and C) according to sequence variations (Lee et al. 2007). Due to the large number of serotypes the identification of individual strains is rarely undertaken due to the time and costs involved. Rhinoviruses are most commonly detected during the autumn and spring, but are associated with infection all year round (Monto 2002).

1.1.3 Influenza virus

Influenza virus was one of the first viruses to be detected and results in yearly epidemics in temperate climates and occasionally in worldwide pandemics. Data from the general practice surveillance network in England and Wales between 1967 and 2006 demonstrated peaks of infection occurred over the winter months and depending on the exact strain of influenza virus, either adults or children were more commonly affected in various years (Fleming & Elliot 2008). There are three subtypes of influenza (A, B and C) with subtypes A (FA) and B (FB) being structurally almost identical and subtype C being slightly different (Mahony 2008). The FA virion is studded with glycoprotein spikes of haemagglutinin (HA) and neuraminidase (NA), in a ratio of approximately four to one, projecting from a host cell-derived lipid membrane. A smaller number of matrix (M2) ion channels traverse the lipid envelope. The envelope and its three integral membrane proteins HA, NA, and M2 overlay a matrix of M1 protein, which encloses the virion core. Internal to the M1 matrix are found the nuclear export protein (NEP, also called non-structural protein 2, NS2) and the

ribonucleoprotein complex, which consists of the viral RNA segments coated with nucleoprotein and the heterotrimeric RNA-dependent RNA polymerase (Bouvier & Palese 2008). FA viruses are further characterized by the subtype of HA and NA. Those proteins mutate naturally over time resulting in 'antigenic drift' and global pandemics. The organization of the FB virion is similar to that of FA with four envelope proteins: HA, NA, and, instead of M2, NB and BM2 (Bouvier & Palese 2008). Influenza C virions are structurally very different from those of FA and FB with only one major surface glycoprotein, the hemagglutinin–esterase-fusion protein, which is functionally similar to the HA, and NA and one minor envelope protein (Bouvier & Palese 2008).

1.1.4 Parainfluenza virus

Parainfluenza (PF) virus possesses at least one non-structural and six structural proteins (fusion protein, haemagglutinin neuraminidase protein [HN], matrix protein, nucleoprotein, phosphoprotein and large polymerase protein), which, with the exception of the HN protein, are very similar structurally and functionally to those present in RSV (Hall 2001). There are four subtypes of parainfluenza (PF1-4), with PF4 being less common than the subtypes PF1, PF2 and PF3 (Mahony 2008). An epidemiological study (Laurichesse et al. 1999) of the parainfluenza virus including data from England and Wales between 1975-1999 demonstrated PF1 and PF2 had peaks of infection in alternate years, whereas PF3 and PF4 had yearly infection peaks. PF1, PF2 and PF4 were most commonly reported in the

winter months (October – January) whereas PF3 was most commonly reported between April and August. PF3 and PF4 were more commonly reported in infants than PF1 and PF2 (Laurichesse et al. 1999).

1.1.5 Adenovirus

Adenoviruses (AdV) are non-enveloped and 65 to 80 nanometres (nm) in diameter. The virion has 20 triangular faces and 12 vertices, with 240 hexons and 12 pentons. Each penton consists of a base and a fibre with a terminal knob. Inside the capsid is a single molecule of double-stranded DNA. Over 50 serotypes of human AdVs have been recognized and grouped into six species (A-F) on the basis of their haemagglutinating properties, biophysical and biochemical criteria and DNA sequence homology (Nazir & Metcalf 2005). Although all six species can cause all common types of illness (e.g. respiratory, gastrointestinal, conjunctivitis) associated with AdV, one large study (over 3000 samples) showed that the C species appeared to more commonly cause respiratory illness in children less than five years of age than the other species (Cooper et al. 2000). There were also marked differences in seasonality within and between the six species with some serotypes being more common in the spring and summer and others in the winter months (Cooper et al. 2000).

1.1.6 Parechovirus

Parechoviruses (PEV), like HRV, are from the *picornaviridae* family (Table 1.1) thus their structure is very similar to that of HRV (Stanway et al. 2000). PEVs have recently been reclassified and there are at least six different types (PEV 1-6) (Harvala et al. 2008). PEV2 is rarely detected in humans, but the other five types have been associated with respiratory tract infections in young children (with PEV1 and PEV6 being the most common), although all PEV types are also commonly found in nasal secretions of children without respiratory symptoms (Harvala et al. 2008). A UK study of respiratory samples taken in 2007 demonstrated peaks of PEV infection occurred in late summer (July and August) and early winter (November and December) (Harvala et al. 2008). A large study from the Netherlands between 2000-2007 (van der Sanden et al. 2008) demonstrated PEV1 was most commonly detected in the autumn and winter months, whereas PEV3 was mainly detected in the spring and summer. No seasonality could be identified for the other PEV types due to small numbers of positive samples. A periodicity was also noted; for PEV1 high peaks of infection occurred yearly, whereas high peaks of PEV3 infection occurred in alternate years (van der Sanden et al. 2008). It must be noted that this study (van der Sanden et al. 2008) included samples from multiple anatomical sites (i.e. respiratory secretions, stool samples, cerebrospinal fluid) rather than just respiratory samples as in the study from the UK (Harvala et al. 2008), which might have impacted on the epidemiological findings.

1.1.7 Enterovirus

Enteroviruses (EV), as HRV and PEV, are classified in the *picornaviridae* family (Table 1.1) and have a similar structure. There are over 60 subgroups and they commonly cause respiratory illness in infants and young children, although they also are found in asymptomatic individuals (Mahony 2008). EV demonstrate a similar seasonality to HRV, causing respiratory infections most commonly in the spring and autumn (Jacques et al. 2008).

1.1.8 Human Metapneumovirus

Human metapneumovirus (HMPV) was first detected in 2001 from infants and children with respiratory tract symptoms in the Netherlands and is classified in the *metapneumovirus* genus (Table 1.1). It consists of nine proteins (nucleocapsid RNA binding protein, nucleocapsid phosphoprotein, non-glycosylated matrix protein, fusion glycoprotein, transcription elongation factor, RNA synthesis regulatory factor, small hydrophobic surface protein, major attachment protein and a major polymerase subunit) (Biacchesi 2003) and consists of two genetically distinct subgroups (A and B) with each subgroup divided further into another two groups (A1, A2 and B1 and B2) (Mackay et al. 2004). A 25 year longitudinal study in the USA retrospectively tested respiratory samples for HMPV from previously healthy children less than five years of age and found peaks of infection occurred in late winter coinciding with the second half of the RSV season (Williams et al. 2004).

1.1.9 Human Bocavirus

Human Bocavirus (HBOV) was first detected in 2005 from randomly selected respiratory samples from children and adults in Sweden (Allander et al. 2005). It is from the *Parvoviridae* family, although has not been formally classified (Table 1.1). HBOV is a small, non-enveloped virus with an isometric nucleocapsid that contains a single molecule of linear, single-stranded DNA; the complete genome length has not been determined. The genome encodes for the non-structural proteins (NS1 and NP1) and two viral capsid proteins (VP1 and VP2) although the function of these proteins are currently unknown (Schildgen et al. 2008). Although HBOV is frequently detected in respiratory samples, there is some debate as to whether it is pathogenic, as it is frequently found in asymptomatic children (Martin et al. 2009) and respiratory samples positive for HBOV are also often positive for another respiratory virus (e.g. RSV) (Choi et al. 2006; Manning et al. 2006). The seasonality of HBOV has yet to be fully elucidated, but may be more prevalent in the winter and spring months (Schildgen et al. 2008).

1.2 Pathology

The pathological findings in acute bronchiolitis were initially demonstrated at autopsy by Hubble and Osborn (Hubble & Osborn 1941). RSV infection results in acute inflammation mainly involving the medium and small bronchioles (less than 150μm in diameter) (Johnson et al. 2007). Inflammatory infiltrates are symmetrical and circumferential in the small

bronchioles and larger airways, but at the level of the terminal bronchioles are often asymmetrical and more clearly centred around the pulmonary arteriole. Airway lumens are occluded by epithelial sloughing, macrophages, fibrin and mucus and inflammatory cells aggregate around the bronchial arteries resulting in dilatation and congestion (Johnson et al. 2007). Mechanical obstruction, therefore, of the small airways appears to be important in the pathogenesis of RSV disease (Johnson et al. 2007). The infant lung has smaller calibre airways and very poor collateral ventilation with little communication between neighbouring alveoli (Aherne et al. 1970). In comparison, the adult lung has good collateral ventilation allowing a sufficiently high pressure to build up distal to an occluding plug to lead to its expulsion, however, this appears not to happen in the infant lung (Aherne et al. 1970).

Histological evidence of recovery from bronchiolitis, by studying ciliated epithelial cells from nasal epithelial biopsies, becomes apparent within days after the onset of symptoms, although complete resolution can take several months (Wong et al. 2005). Symptoms may persist for several weeks after the acute infection has cleared (Hall 2001).

Aherne et al and Ferris et al demonstrated similar histopathological findings at autopsy in infants with bronchiolitis regardless of the viral aetiology (RSV, adenovirus, parainfluenza, influenza or rhinovirus), suggesting all viruses lead to a similar pathological process (Aherne et al. 1970; Ferris et al. 1973).

1.3 Clinical presentation of RSV

RSV infection affects children in epidemics with marked seasonality. In temperate climates, in both the northern and southern hemispheres, there are epidemics of RSV in the winter, whereas in the tropics there are epidemics during the hot and rainy seasons (Shek & Lee 2003). At extremes of temperature RSV activity is more continuous throughout the year (Welliver 2007). Humidity and ultraviolet B (UVB) radiation have been linked with susceptibility to symptomatic RSV infection, with higher levels of humidity and lower levels of UVB increasing vulnerability (Welliver 2007; Yusuf et al. 2007).

RSV transmission occurs by two routes; large particle or droplet formation or via direct inoculation from fomites, into the eyes or nose, such that close contact (proximity of less than six feet) is required for the spread of the virus (Hall & Douglas 1981).

Once the virus infects the upper respiratory tract there is an incubation period of two to eight days in which RSV replicates in the nasopharyngeal epithelium, often causing coryzal symptoms. It then spreads to the lower respiratory tract one to three days later (Hall 2001). LRTIs are characterised by cough, dyspnoea and cyanosis. Clinical examination may reveal prolonged expiration, wheeze, crackles and signs of respiratory distress (Hall 2001). Infants may require nasogastric feeding or intravenous fluids. Infants may also present with apnoea, which is more common in prematurely born

infants, those with BPD (Meert et al. 1989) and infants less than three months of age (Ralston & Hill 2009).

1.4 Mortality

Mortality from bronchiolitis (any virus) in infants older than 28 days of age considerably diminished in England and Wales between 1979 and 2000 (from 21.5 to 1.8 per 100,000) (Panickar et al. 2005), with RSV being the virus that caused the highest rates of mortality. Fleming et al estimated that in the winters in England between 1989 and 2000 there were a total of 185 (2.9 per 100,000) deaths from RSV bronchiolitis and 129 (2.0 per 100,000) deaths from influenza virus in infants between one and 12 months of age (Fleming et al. 2005). Those data are similar to those from the USA between 1979 and 1997 which highlighted a mortality rate of approximately 1.5 per 100,000 for bronchiolitis (any virus) in infants less than one year of age and between 171 and 510 RSV-associated deaths in children less than five year of ages, with 71% occurring in infants less than one year of age (Shay et al. 2001). Deaths peaked in January (18%) and most (76%) occurred during the typical November to April RSV season. Another study (Thompson 2003) in the USA comparing RSV and influenza infections between 1990 and 1999 highlighted mortality rates of 5.4 and 2.2 per 100,000 person-years for RSV and influenza respectively in infants less than one year of age.

1.5 Hospitalisation

In 2002, in the USA, there were an estimated 149,000 patients hospitalised with bronchiolitis (any virus) accounting for approximately 491,700 inpatient days per year, with a total annual cost of $US 543 million and a mean cost of $US 3799 per patient (Pelletier et al. 2006). Although any of the common respiratory viruses can cause disease severe enough to warrant hospitalisation, the majority of hospitalisations are due to RSV. One study estimated approximately 27,900 children aged less than four years of age were hospitalised every year in England and Wales due to RSV compared to only 4200 due to influenza (Pitman et al. 2007). A prospective study of 1429 previously well children less than five years of age followed between 1974 and 1993 as part of a vaccine evaluation programme, found only two children (1.4 per 1000 children) were admitted for a LRTI with an NPA positive for Parainfluenza (one PF1 and one PF3) (Reed et al. 1997).

Another study estimated rates of hospitalisation during times of high influenza, but low RSV, prevalence in the community and found hospitalisation rates for acute respiratory disease of 16-19 per 100,000 person years in previously well children less than two years of age (Izurieta et al. 2000). This increased to 64-98 per 100,000 person years for children less than two years of age with high risk conditions (including prematurity and chronic respiratory and cardiac conditions) (Izurieta et al. 2000). A limitation of that study (Izurieta et al. 2000) was the lack of virologically confirmed illness in the infants.

Amongst infants and young children up to 56 months of age hospitalised for viral LRTIs, those with HMPV were compared to those with RSV (Mullins et al. 2004). Infants with HMPV were older (median age of hospitalisation 11.5 versus 3 months, $p<0.001$) and had more underlying medical conditions (54% versus 29%, $p<0.05$). Most children in the HMPV group had asthma as an underlying condition (8 out of 14). There were no significant differences in the other clinical characteristics of the two groups (Mullins et al. 2004). A study of children less than three years of age attending an emergency department compared the presentations of the children infected with HMPV, RSV and HRV (Manoha et al. 2007). Significantly more infants with RSV infection were hospitalised as a result of their infection than those with HMPV (67% versus 47%, $p<0.02$) and of those hospitalised, those with HMPV had higher rates of asthma than those with RSV (25% versus 4%, $p<0.02$). Infants with RSV were significantly more likely to present with cough, pharyngitis, bronchitis and feeding difficulty than infants with HRV (Manoha et al. 2007). Calvo et al compared children less than 14 years of age infected with HBOV to those infected with RSV, AdV and HRV (Calvo et al. 2008). Children developing HBOV were older than those with RSV (20.8 versus 9.8 months, $p=0.017$) and were less likely to be hospitalised with a diagnosis of bronchiolitis (32% versus 65%, $p<0.0001$). In addition, children with HBOV had a higher leucocyte count and higher serum C reactive protein level (Calvo et al. 2008).

Apart from the type of virus affecting an individual there are also multiple risk factors for hospitalisation with RSV bronchiolitis (Table 1.2).

Table 1.2: Risk factors for hospitalisation with RSV bronchiolitis

Reference	Risk factor for hospitalisation
(Hall et al. 2009)	Prematurity
(Cilla et al. 2006)	Birth weight <2500g
(Simon et al. 2007)	BPD
(Duppenthaler et al. 2004; MacDonald et al., 1982)	Congenital cardiac disease
(Hall et al. 1986)	Immunodeficiency
(Wilkesmann et al. 2007)	Neurological impairment
(Simoes, 2003)	Male gender
(Simoes, 2003)	Young age (<6 months)
(Simoes, 2003)	Birth in the first half of the RSV season
(Simoes, 2003)	Crowding/siblings
(Simoes, 2003)	Attendance at day-care
(Bradley et al. 2005)	Maternal smoking
(Ségala et al. 2008)	Air pollution

1.6 Long term respiratory morbidity following viral infections in term born infants

1.6.1 Follow up of infants hospitalised with viral LRTIs

A study following up infants previously hospitalised for RSV LRTI found that at eight years of age they had experienced more episodes of wheeze since discharge and had reduced lung function (lower peak expiratory flow rate [PEFR] and $FEV_{0.75}$/VC) compared to an age, sex and social class matched group (Sims et al. 1978). Another study investigating infants ten years after hospitalisation for RSV LRTI showed significantly more of the RSV group compared to the controls had a history of cough (9% versus 2%) and wheeze (42% versus 19%), significantly lower lung function (reduced PEFR, FVC, FEV_1, FEV_1/FVC, MEF_{50}, MEF_{75} and MEF_{25-75}) and evidence of increased bronchial lability after either an exercise or histamine challenge (Pullan & Hey, 1982). A limitation of both of those studies (Sims et al. 1978; Pullan & Hey 1982) was that the control groups were not contemporaneously recruited and followed.

Two longitudinal studies have recruited subjects and controls simultaneously (Sigurs et al. 1995; Korppi et al. 2004). Sigurs et al (1995) demonstrated that at three years of age the RSV group had more asthma (23% versus 1%, $p<0.001$) and atopy as assessed by either skin prick testing or serum IgE to various allergens (32% versus 9%, $p=0.002$) suggesting RSV LRTI was involved in the development of asthma and atopy. At seven years of age, the

RSV group had significantly higher rates of asthma diagnosis than the control group (30% versus 3%, p<0.001) (Sigurs et al. 2000). In addition, RSV bronchiolitis was a significant risk factor for the development of allergic sensitisation (odds ratio of 2.4) (Sigurs et al. 2000). At 13 years of age the RSV group had more current asthma or wheeze (43% versus 8%, p<0.001), reduced baseline lung function (FEV_1, FEV_1/FVC, FEF_{25}) and a greater fall in FEV_1 on challenge testing (6.1% versus 4.6%, p=0.047) than the control group (Sigurs et al. 2005). In another prospectively followed cohort, young adults followed for 20 years after RSV LRTI in infancy were found to have similar rates of doctor-diagnosed asthma, but increased lung function abnormalities, that is reduced FEV% and MEF_{25}, independent of atopy, compared to the control group (Korppi et al. 2004).

One study has investigated the risk of chronic respiratory morbidity after hospitalisation with HMPV infection (García-García et al. 2007). HMPV bronchiolitis in infancy was associated with an increased risk of an asthma diagnosis (OR 15.9) at five years of age, and that this risk was greater than that for RSV (OR 10.1) (García-García et al. 2007).

Table 1.3 gives the results of previous lung function studies following viral LRTIs in previously healthy infants and children. It demonstrates viral LRTIs in infancy, mainly infants requiring hospitalisation for RSV infection, are associated with abnormal lung function from immediately after the acute infection into early adulthood. This raises the questions of whether viral LRTIs other than RSV are similarly associated with abnormal lung function at

follow up and whether infants with underlying conditions (e.g. prematurely born infants) are similarly affected.

Table 1.3: Results of previous lung function studies following viral LRTI in previously healthy infants and children (Updated from PhD thesis by S. Broughton, 2009)

Author	Age at Infection	RSV proven	Method of RSV identification	Wheeze required for diagnosis	Hospitalised	Age at lung function testing	Index group (n)	Control Group (n)	Lung function tests	Results
(Stokes et al. 1981)	<151 days	50% (5% AdV)	Not stated	No	Yes	1-18 days and 13 months after discharge	22	0	Plethysmography, forced oscillation (inspiratory & expiratory resistance)	74% hyperinflated (TGV >40ml/kg) at 1-18 days, 39% hyperinflated by 13 months.
(Merth et al. 1996)	<6 months	88%	DFA	No	Yes	2 weeks, 3 and 12 months	24	69	FRC_{He}, C_{rs}	No significant differences
(Seidenberg et al. 1989)	4-41 weeks	Yes	DFA	Yes	Yes	4 months after admission	14	6	C_{rs}, R_{rs}, V_{max} FRC and FRC_{pleth}	Elevated TGV
(Tepper et al. 1992)	Mean age 5.2 months	No	Not done	Yes	50%	8 and 14 months	18	24	V_{max} FRC and PC_{30}	Lower V_{max} FRC and PC_{30} at 8 and 14 months
(Caswell et al. 1990)	<21 weeks of age	60%	DFA or viral culture	Not stated	Yes	9 weeks and 8 months after discharge	13	0	Plethysmography, V_{max} FRC and PD_{30}	15% hyperinflated (TGV>120% predicted), 40% raised R_{aw} (>140% predicted) and 100% reduced V_{max} FRC at 9 weeks. 8% hyperinflated, 24% raised R_{aw} at 8 months
(Dezateux et al. 1997)	6.2-19.7 weeks	Yes	Not stated	No	Yes	11 months	29	29	FRC_{pleth}, R_{aw}, C_{rs}, tPTEF/tE	Decreased tPTEF/tE
(Henry et al. 1983)	1-13 months	52% (3% AdV)	Not stated	No	Yes	At discharge, 3 and 12 months after discharge	93	0	FRC_{pleth}, R_{aw}, sG_{aw}	77% hyperinflated (TGV >40 mL/kg) at discharge, 43% at 3 months, 17% at 1 year
(Young et al. 1995)	<1 year	20%	Not stated	Not stated	20% (both RSV)	1 year	10	236	V_{max} FRC, C_{rs}, R_{rs}, T_{me}/T_e and PC_{40}	No significant differences

(Henry et al. 1983)	1-12 months	49% (2% AdV)	'viral studies'	No	Yes	2 years after infection	40	0	FRC_{pleth} and forced oscillometry	55% hyperinflated (TGV>120% predicted)
(Murray et al. 1992)	<1 year	68%	DFA or viral culture	No	Yes	6 years	73	73	Spirometry, PEFR, PC_{20}	Increased PC_{20}
(Sly & Hibbert 1989)	2-42 weeks	100%	DFA & viral culture	No	Yes	6 years	25	0	Spirometry, PD_{20}	Lower FEV_1/VC (16%)
(Poulsen et al. 2006)	0.9 years	100%	DFA & RSV Ab assay	No	Some	6.8 years	78	78	FEV_1, PEFR	Lower FEV_1 and PEFR
(Hall et al. 1984)	<24 months	Yes	Viral culture	No	Yes	7 (6-8) years	23	0	Spirometry, PEFR	Lower FEF_{25-75}
(Mok & Simpson 1982)	<12 months	50%	DFA or viral culture	No	Yes	7 years	200	200	Spirometry, PEFR, forced oscillometry	Lower PEFR, $FEV_{0.75}$, FEV_1, FEV_1/FVC and FEF_{25-50}
(Cassimos et al. 2008)	<1 year	100%	DFA	No	Yes	7.5 (4.0-9.5) years	189	60	Spirometry, PEFR	Lower FEV_1, FEF_{50} and PEFR
(Fjaerli et al. 2005)	<1year	61%	DFA	No	Yes	7.7 (7.2-8.5) years	57	64	Spirometry	Lower FEV_1 and FEF_{50}
(Juntti et al. 2003)	<1 year	100%	DFA or latex agglutination test	No	Yes	8 (6.9-10.3) years	38	44	Spirometry, impulse oscillometry	No significant differences
(Sims et al. 1978)	"infancy"	Yes	Viral culture	No, but 95% had wheeze	Yes	8 years	35	35	Spirometry, PEFR	Lower PEFR and $FEV_{0.75}$/VC
(Gurwitz et al. 1981)	2-23 months	No	Not done	Yes	Yes	9-12 years	48	0	Spirometry, plethysmography, PEFR	Lower VC, FEV_1, MEF_{25-75}, PEFR, raised TLC and RV/TLC
(Noble et al. 1997)	1-11 months	66%	DFA	No	Yes	10 years	61	47	Spirometry, PEFR, plethysmography	Lower PEFR, $FEV_{0.75}$, FEV_1, FEF_{25-75} and higher R_{aw}
(McConnochie et al. 1985)	24 months	No	Not done	Yes	4% (1 infant)	10 (8-12) years	25	25	Spirometry, PEFR	Lower FEF_{25-75}
(Pullan & Hey, 1982)	<12 months	Yes	Not stated	Not stated	Yes	10 (8.9-11.5) years	98	104	Spirometry, PEFR, single breath nitrogen wash-out	Lower PEFR, FVC, FEV_1, FEV_1/FVC, MEF_{50}, MEF_{75}, MEF_{25-75}
(Turner et al.	<2 years	Yes	Not stated	No	2 infants	11 years	16	159	Spirometry	Reduced FEF_{25-75}

2002)										
(Kattan et al. 1977)	<18 months	No	Not done	Yes	Yes	11 years	23	0	Plethysmography, PEFR, Spirometry	41% elevated RV/TLC.
(Stein et al. 1999)	<36 months	Yes	Not stated	No	No	11 years	110	189	FEV_I	Reduced FEV_1
(Hyvärinen et al. 2007)	<2 years	30%	DFA, RSV Ab assay and RT-PCR	Yes	Yes	11.4 (10.3-12.3) years	80	0	Spirometry, PD_{20}	33% had an abnormal spirometry result, 23% abnormal PD_{20}
(Sigurs et al. 2005)	<1 year (91% ≤6 months)	100%	EIA	No	Yes	13.4 (13.0-14.0) years	47	93	Spirometry	Lower FEV_1/FVC and FEF_{75}
(Korppi et al. 2004)	<2 years	100%	DFA, RSV Ab assay	No	Yes	19 (18-20) years	36	45	Spirometry, PD_{20}	Lower FEV_1/FVC and MEF_{25}
(Larouche 2000)	<18 months	No	Not done	No	Yes	21 (17-30) years	42	42	Spirometry, PC_{20}	Lower FEV_1/FVC, PC_{20}

Key for Table 1.3:

C_{rs}: Compliance of the respiratory system

DFA: Direct fluorescent-antigen test

EIA: Enzyme immunoassay

FEF_{25-50}: Forced expiratory flow from 25-50% of vital capacity

FEF_{25-75}: Forced expiratory flow from 25-75% of vital capacity

FEF_{50}: Forced expiratory flow at 50% of vital capacity

FEF_{75}: Forced expiratory flow at 75% of vital capacity

$FEV_{0.75}$: Forced expiratory volume at 0.75 seconds

FEV_1: Forced expiratory volume at one second

FEV_1/FVC: Ratio of forced expiratory volume at one second to forced vital capacity

$FEV_{0.75}$/VC: Ratio of forced expiratory volume at 0.75 seconds to vital capacity

FEV_1/VC: Ratio of forced expiratory volume at one second to vital capacity

FRC_{He}: Functional residual capacity measured by helium gas dilution

FRC_{pleth}: Functional residual capacity measured by plethysmography

MEF_{25-75}: Maximal expiratory flow from 25-50% of vital capacity

MEF_{50}: Maximal expiratory flow at 50% of vital capacity

MEF_{75}: Maximal expiratory flow at 75% of vital capacity

$PC_{20, 30, 40}$: Bronchial responsiveness to a provocation challenge

$PD_{20, 30}$: Bronchial responsiveness to a provocation challenge

PEFR: Peak expiratory flow rate

R_{aw}: Airways resistance

R_{rs}: Resistance of the respiratory system

RSV Ab test: RSV antibody test

RT-PCR: Reverse transcriptase polymerase chain reaction

RV/TLC: Ratio of residual volume to total lung capacity

sG_{aw}: Specific airways conductance

T_{me}/T_e: Ratio of time to maximum expiration to total expiratory time

TGV: Thoracic gas volume

TLC: Total lung capacity

tPTEF/tE: Ratio of time to peak tidal expiratory flow to total expiratory time

VC: Vital capacity

V_{max} FRC: Maximal flow at functional residual capacity

1.6.2 Community based studies

Young et al found no significant differences in lung function results at one year of age between those infants who had and had not had viral LRTIs (Young et al. 1995). At two years of age, however, the infants who developed bronchiolitis had increased rates of asthma and more had had a wheezy episode (Young et al. 1995). The cohort was seen at 11 years (Turner et al. 2002) and after adjustment for factors known to affect lung function, the bronchiolitis group compared to the control group, had reduced FEF_{25-50} and more of them had wheezed at some point since their bronchiolitic episode. In a study of 50 infants, the 25 with viral bronchiolitis (no virology reported) in infancy had reduced FEF_{25-50} at 11 years of age compared to a control group of age matched children, but there was no difference in the use of anti asthma medication (McConnochie et al. 1985). Similarly, the Tucson Children's Respiratory Study (Stein et al. 1999) found reduced FEV_1 at 11 years of age in those who had had RSV LRTI in the first three years after birth compared to a similarly prospectively followed control group. The RSV group had more wheeze with an odds ratio (OR) of 2.4 for frequent wheeze and an OR of 1.7 for infrequent wheeze compared to the no LRTI group. The COAST study (Lemanske et al. 2005) demonstrated, in a high risk cohort, that is with at least one atopic parent, that HRV bronchiolitis in infancy was associated with an increased risk of subsequent wheezy episodes at three years of age (OR 3.6). Those data (Young et al. 1995; McConnochie et al. 1985; Stein et al. 1999; Turner et al. 2002; Lemanske et al. 2005) would suggest that even viral LRTIs not severe enough to warrant hospitalisation

are still associated with chronic respiratory morbidity and that these infants may, therefore, have benefitted from prophylaxis. In addition, those data (Young et al. 1995; McConnochie et al. 1985; Stein et al. 1999; Turner et al. 2002; Lemanske et al. 2005) imply that infants developing milder viral LRTIs still use health care resources after the acute episode and therefore cost-benefit analysis studies should include these infants as well as those with more severe disease.

1.6.3 Lung function after other viral LRTIs

Few studies have investigated the effect of viruses other than RSV on subsequent lung function in children. Fjaerli et al found no significant differences in FVC, FEV_1 or FEF_{50} between infants hospitalised for RSV LRTI or RSV negative LRTI at seven years of age (Fjaerli et al. 2005). Mok and Simpson found no significant differences in PEFR, FEV_1, FVC, FEV_1/FVC, FEF_{25-50} or R_{rs} at seven years of age between infants who had been admitted for either RSV LRTI or RSV negative LRTI (Mok & Simpson 1982). Hyvarinen et al demonstrated that at 11 years of age infants who had been hospitalised for RSV negative bronchiolitis (adenovirus, influenza A and B, parainfluenza 1-3, rhinovirus, enteroviruses, coronaviruses or no virus detected) had significantly better lung function (FVC and FEV_1/FVC) than the RSV positive group (Hyvärinen et al. 2007).

The Tucson Children's Respiratory Study (Stein et al. 1999) found no association with mild, that is not requiring hospitalisation, parainfluenza or

other viral LRTIs (adenovirus, influenza, rhinovirus, cytomegalovirus) in infancy and FEV_1 at 11 years of age. They did, however, note that infants who developed other LRTIs, but had negative microbiological studies, had significantly lower FEV_1 than infants who did not develop a LRTI.

In a study of forty four 18-20 year olds prospectively followed from hospitalisation for bronchiolitis in infancy, no significant differences were found in FEV_1, FEV%, MEF_{50} or MEF_{25} between infants hospitalised for RSV bronchiolitis or non-RSV bronchiolitis (influenza A or B, parainfluenza 1-3, adenovirus or negative for all viruses, hospitalised outside the RSV season) (Piippo-Savolainen et al. 2007). Simila et al investigated children who had had adenovirus type 7 LRTI between the ages of three months and seven years and were followed to 9-12 years of age (Simila et al. 1981). Seventy three percent of the children were shown to have abnormal FVC and FEV_1 suggesting adenoviral LRTIs were associated with subsequent reduced lung function.

Those data suggest viruses other than RSV may be associated with impaired lung function at follow up in term born infants. Thus, an aim of this thesis was to test the hypothesis that other viruses, as well as RSV, adversely impacted on the lung function of prematurely born infants.

1.7 Prematurely born infants and chronic respiratory morbidity after viral LRTIs

1.7.1 Symptoms

Infants born prematurely and developing RSV LRTIs are more likely to have symptoms of chronic respiratory morbidity (cough and wheeze) at follow up than similar infants not developing RSV LRTI (Broughton et al. 2005). Broughton et al in a prospectively followed cohort of infants born prior to 32 weeks of gestation, found infants with RSV LRTI had significantly more and longer hospitalisations and more GP attendances in the first year after birth than infants who had either no LRTI or a LRTI where no virus was detected ('no virus' group) (Broughton et al. 2006). The HMPV group had the highest proportion of hospitalisations and longest mean length of hospitalisation. The RSV group had more cough and wheeze than the no virus group, and more wheeze than the HRV group. Multivariate analysis demonstrated HMPV to be a risk factor for hospitalisation, GP attendance and wheeze at follow up (Broughton et al. 2006).

1.7.2. Lung function

RSV LRTI has been shown to be associated with abnormal lung function at follow up in prematurely born infants. In a cohort of infants born prior to 32 weeks of gestation those developing RSV LRTI (either in the community or requiring hospitalisation) had significantly higher airways resistance (R_{aw}) than the infants who did not have a RSV LRTI at one year corrected age

(Broughton et al. 2007). Regression analysis demonstrated HMPV to also be associated with increased R_{aw} at follow up (Broughton et al. 2007). Those data suggest other viruses as well as RSV impact on subsequent lung function in prematurely born infants and an aim of this thesis was to test that hypothesis.

1.8 Viral identification techniques

1.8.1 Conventional techniques

The gold standard for the identification of respiratory viruses has historically been viral culture, which involves infecting cells that the virus being tested is know to affect and then observing for any cytopathic effects within those cells. One of the major disadvantages of this technique is that it is usually too slow for routine clinical practice. Another conventional technique is the direct immunofluorescence assay where a monoclonal antibody joined to a fluorophore is directed against an antigen in the virus. When the antibody binds to the antigen the fluorescence produced can be visualised under a microscope. The advantage of this technique is that the turnaround time is much faster, meaning it can be used in routine clinical practice (Freymuth et al. 2006).

1.8.2 Polymerase chain reaction (PCR)

PCR was first described by Saiki et al for the prenatal diagnosis of sickle cell anaemia (Saiki et al. 1985) and it has subsequently been used for viral identification (Paton et al. 1992).

PCR utilises a pair of primers that hybridise to two strands of a DNA target across a defined region that becomes exponentially amplified. The hybridised primers act as a substrate for a DNA polymerase to create two complementary strands through the addition of deoxynucleotides. PCR usually involves three steps:

1. Denaturation: heating the reaction to melt the DNA, producing single-stranded DNA molecules.
2. Annealing: Cooling the reaction to allow annealing of the primers to hybridise to the single-stranded DNA template.
3. Extension: DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding deoxynucleotides complementary to the template in 5' to 3' direction.

RNA can also be amplified by the addition of a pre-PCR reverse transcriptase step. A complementary DNA (cDNA) strand is initially produced, by reverse transcriptase (RT), and the cDNA then undergoes PCR. Therefore, PCR can detect DNA viruses and RT-PCR RNA viruses. Traditional detection and analysis of PCR products requires agarose gel

electrophoresis in the presence of ethidium bromide with visualisation by illumination with short wave UV light (Hibbitts & Fox 2002).

1.8.3 Real-time PCR

Real-time PCR was developed by Higuchi et al enabling the amplification and detection of the PCR product to occur in a single reaction tube thus eliminating the expensive and time consuming post-PCR steps and also increasing the sensitivity and specificity of the test (Higuchi et al. 1992). In real-time PCR a fluorescent reporter dye binds to the PCR product that is formed and generates a fluorescent signal that reflects the amount of product formed. One of the main fluorescence-monitoring systems for DNA amplification are hydrolysis probes, which include TaqMan probes (Heid et al. 1996). TaqMan probes are dual labeled oligonucleotides containing a fluorescent dye on the 5' base, and a quenching dye on the 3' base. When irradiated, the excited fluorescent dye transfers its energy by Förster resonance energy transfer (FRET) to the nearby quenching dye molecule. The close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. When the polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the 5’ end of the probe which contains the reporter dye. This releases the reporter dye from quenching and ends the activity of the quencher and the reporter dye starts to emit fluorescence which increases in proportion to the rate of probe cleavage and hence product formation.

Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye.

Real time PCR also allows several PCR products (usually to a maximum of four) to be identified simultaneously ('real-time multiplex PCR'), providing each product gives off a different fluorescent signal (Wittwer et al. 2001); this saves time and costs.

PCR has been shown to be more sensitive and specific for viral detection than conventional methods (Falsey et al. 2002). One study in children found multiplex PCR resulted in a 34% increase in respiratory virus identification over direct fluorescence antibody (DFA) alone and 20% over DFA combined with viral culture. Multiplex PCR identified 14% more RSV cases than DFA, 10% more RSV cases than DFA and viral culture, and four times as many (23 versus 91 infants) HRV cases than DFA (Freymuth et al. 2006). As a consequence in this thesis, respiratory samples will be tested using real-time multiplex PCR.

1.9 Treatment of acute bronchiolitis and RSV LRTI

Treatment for acute bronchiolitis (RSV or other viral causes) is mainly supportive with oxygen supplementation and ensuring adequate hydration, via either nasogastric feeding or intravenous fluids. No single medication has been shown to improve clinical outcomes.

1.9.1 Bronchodilators

Many studies have investigated the effect of salbutamol or ipratropium bromide on the clinical course of bronchiolitis. A systematic review (Gadomski & Bhasale 2006) demonstrated that although bronchodilators (including salbutamol and ipratropium bromide) caused significant improvements in clinical scores they did not improve oxygen saturations, reduce hospital admission after outpatient treatment, shorten the duration of hospitalization or reduce the time to resolution of illness at home.

1.9.2 Adrenaline

Adrenaline has bronchodilator properties, but also decongests the nasal mucosa by reducing oedema. A meta-analysis of 14 randomised controlled trials comparing adrenaline with either placebo or another bronchodilator demonstrated that adrenaline improved short term clinical scores, but did not reduced rates of hospitalisation (Hartling et al. 2004).

1.9.3 Corticosteroids

A systematic review (Patel et al. 2004) demonstrated corticosteroids alone did not improve clinical scores, reduce hospital admissions or length of stay in hospital. A small study of 48 term and 13 prematurely born infants between three and 12 months of age found nebulised dexamethasone and adrenaline resulted in a reduction in length of hospital stay compared to

nebulised adrenaline and 0.9% saline (9.1 versus 6.5 days, p=0.018), but only in the prematurely born infants (Bentur et al. 2005).

1.9.4 Hypertonic saline

Hypertonic saline may have some benefit in treating infants with acute bronchiolitis. In a meta-analysis, Zhang et al showed hypertonic (3%) saline improved clinical scores by between 0.75 (11.2%) and 1.28 (20%) points (compared to 0.9% saline) and reduced length of hospital stay by one day (25.9%) (Zhang et al. 2008). In one study (Kuzik et al. 2007) from the above meta-analysis there was a 26% reduction in the length of stay in hospitalised infants receiving nebulised 3% saline compared to normal (0.9%) saline.

1.9.5 Physiotherapy

In a meta-analysis of three clinical trials, chest physiotherapy was not shown to reduce length of hospital stay, oxygen requirements or improve the clinical scores in infants with acute bronchiolitis (Perrotta et al. 2007).

1.9.6 Recombinant human deoxyribonuclease

Since airway obstruction due to intraluminal mucus plugs is an important pathophysiologic feature of RSV bronchiolitis, a mucolytic agent might be efficacious. In a case series (Merkus et al. 2001), improvement in chest radiograph findings, oxygenation and blood gas results were reported after

recombinant human deoxyribonuclease (rhDNase) administration in five infants with severe atelectasis associated with bronchiolitis. In a randomised trial (n=75) (Nasr et al. 2001) an improvement in chest radiograph scores was shown in infants receiving rhDNase compared to placebo (the excipient of the rhDNase- sodium chloride and calcium chloride), but there was no difference in clinical scores or length of hospital stay. In a larger randomised study of infants with moderate RSV bronchiolitis requiring hospital admission (n=225), but not intensive care, no difference was demonstrated in length of hospitalisation or duration of supplementary oxygen between infants receiving rhDNase or placebo (0.9% saline) (Boogaard et al. 2007).

1.9.7 Leukotriene receptor antagonists

Higher levels of cysteinyl leukotriene in bronchoalveolar lavage fluid are found in infants with acute RSV infection compared to controls (Kim et al. 2006). Nevertheless, in a randomised controlled trial of 55 infants admitted to a general paediatric ward, 77% of who were RSV positive, no significant differences were detected in length of hospitalisation, clinical score or cytokine levels between infants receiving a leukotriene receptor antagonist (montelukast) or placebo (Amirav et al. 2008). The study, however, was powered to detect at least a 30% reduction in length of stay (Amirav et al. 2008).

1.9.8 Surfactant

Infants with severe bronchiolitis, that is those requiring intensive care, have been shown to have reduced levels of surfactant (Dargaville et al. 1996); therefore replenishing surfactant from an exogenous source would seem a plausible treatment. Tibby et al in a study of 19 ventilated infants, nine receiving surfactant and ten controls (receiving air as a placebo), showed two doses of bovine surfactant (Survanta) resulted in a reduced deterioration in compliance and resistance of the respiratory system, although there were no differences in duration of ventilation, intensive care or total hospital stay (Tibby et al. 2000). In a randomised trial of 40 infants, the 20 receiving exogenous surfactant (Curosurf) had significantly better blood gas results, higher static compliance and a shorter duration of mechanical ventilation and stay on paediatric intensive care compared to the controls (Luchetti et al. 2002). Both studies included only the most severely unwell infants who had had either no improvement after 12 hours of mechanical ventilation (Luchetti et al. 2002) or were unlikely to remain ventilated for less than 48 hours, based on their oxygenation and ventilation index (Tibby et al. 2000).

1.9.9 Ribavirin

Ribavirin is a virustatic agent which inhibits RSV transcription, glycoprotein expression, syncytium formation and virion release with little cellular toxicity in vitro (Zhang et al. 2003). A systematic review (Ventre & Randolph 2007) of 12 studies including 115 infants who received Ribavirin and 112 controls

concluded that ribavirin did not statistically significantly reduce the length of mechanical ventilation or hospitalisation, but the studies lacked sufficient power to adequately assess those outcomes.

1.9.10 Heliox

Heliox consists of a mixture of helium and oxygen, which has a lower density than air and thus has a reduced resistance to flow in the airways. In addition, carbon dioxide diffuses through helium four to five times faster than through air, which aids ventilation and carbon dioxide removal. In a small randomised controlled crossover trial, use of heliox in non-ventilated infants with mild-moderately severe RSV bronchiolitis was associated with an improved clinical score (Hollman et al. 1998). A more recent prospective, interventional crossover study compared infants with bronchiolitis receiving continuous positive airway pressure (CPAP) ventilation with either air/oxygen or heliox (Martinón-Torres et al. 2008). Those infants receiving CPAP with heliox had a statistically significant improvement in clinical scores and arterial blood gas carbon dioxide results compared to those on CPAP driven with air/oxygen (Martinón-Torres et al. 2008). Two other studies (Gross et al. 2000; Liet et al. 2005), however, found heliox did not result in any improved outcomes. Gross et al (Gross et al. 2000), in ten mechanically ventilated infants, did not demonstrate any statistically significant reductions in ventilation requirements or oxygenation in infants receiving 15 minute periods of varying heliox mixtures (helium proportion varying between 50-70%) compared to those receiving oxygen/nitrogen (50:50) mix. Liet et al in a

small (n=39) multi-centre, randomised, double-blind, placebo controlled trial found no statistically significant differences in clinical score, need for mechanical ventilation or length of stay on paediatric intensive care between infants receiving heliox or placebo (nitrogen/oxygen mix) (Liet et al. 2005).

1.9.11 Prophylaxis

There are currently no safe and effective vaccinations against the viruses that commonly cause bronchiolitis appropriate for use in infants. In the 1960s, a formalin inactivated vaccine against RSV was produced (Kapikian et al. 1969; Kim et al. 1969). In children between two and five years of age the vaccine was not protective as similar numbers of children developed pneumonia in the vaccinated and unvaccinated groups (18% versus 26%). In children aged between 6-23 months, however, the vaccine induced an exaggerated clinical response to naturally occurring RSV infections; 69% of the vaccinated group but only 9% of the control group developed pneumonia ($p<0.0001$) (Kapikian et al. 1969) and 80% of the vaccinated group required hospitalisation at the time of RSV infection, compared to 5% of the control group (Kim et al. 1969).

Passive immunoprophylaxis has been available either as pooled and enriched human immune globulin (RSV-IGIV; Respigam, Medimmune, Gaithersburg, USA) or as a monoclonal antibody (Palivizumab; Medimmune, Gaithersburg, USA). Two randomised controlled trials carried out by the same study group have investigated the use of RSV-IGIV. The first included

101 previously healthy infants born at ≥33 weeks of gestational age and found no difference in length of hospital stay, but a trend towards a reduction in intensive care stay (p=0.06), in the infants receiving RSV-IGIV compared to those receiving the placebo (Rodriguez et al. 1997). The second, the PREVENT trial, included only high risk infants, that is those born less than 35 weeks GA with or without BPD, n=510 (1997). RSV-IGIV reduced hospitalizations by 41% and reduced length of stay by 53%. In addition, infants who received RSV-IGIV required fewer days of supplemental oxygen during their hospital admission. RSV-IGIV was, however, associated with side effects. One percent of all the infants and 13% of those with BPD required diuretics at the time of treatment (1997). RSV-IGIV is administered as an intravenous infusion and could have resulted in fluid overload. In addition, it needed to be given once a month over the RSV season and, as it is human-derived, there is a potential risk of blood-borne infections.

Palivizumab is a humanized monoclonal antibody (IgG) directed against an epitope in the A antigenic site of the F protein of RSV and is about 50-fold more potent than RSV-IGIV (Johnson et al. 1997). It is administered by intramuscular injection and is given monthly over the RSV season. The IMpact RSV study (1998) was a randomised, double-blind, placebo-controlled trial including 1502 prematurely born infants (<36 weeks GA) and demonstrated palivizumab administration resulted in a significant reduction in hospitalisation (4.8% vs. 10.6%, p<0.001), fewer total days of hospitalisation, fewer days of increased oxygen supplementation and fewer intensive care

admissions, although no statistically significant reduction in the need for mechanical ventilation (1998).

Sub group analysis demonstrated infants with BPD had a 39% reduction ($p=0.038$) in hospitalisations compared to the control group (1998). In addition, infants without BPD had a 78% reduction ($p<0.001$), infants born <32 weeks GA a 47% reduction ($p=0.003$) and infants born between 32-35 weeks GA an 80% reduction ($p=0.002$) in hospitalisation compared to the control group (1998).

As palivizumab is a relatively expensive medication (£360 per 50 mg vial) (Royal College of Paediatrics and Child Health 2008) cost benefit analysis studies have been undertaken and not shown palivizumab to be cost effective except in very high risk infants. A systematic review and economic evaluation by the Health Technology Assessment Programme (UK) demonstrated palivizumab may only be cost-effective for children with chronic lung disease when the children had two or more additional risk factors (e.g. age at start of RSV season, siblings at school) (Wang et al. 2008). As a consequence palivizumab is not routinely given to all prematurely born infants. It is, therefore, important to try to identify more accurately which infants will go on to develop RSV and subsequent morbidity in an effort to target prophylaxis to high risk populations and is an aim of this thesis.

There is limited evidence that palivizumab may reduce chronic respiratory morbidity. A non-randomised study followed up 427 infants born at less than 36 weeks GA without BPD. Infants had either received palivizumab or not (the reasons why infants did or did not receive palivizumab were not explored in the study) and were followed for two years with telephone calls or visits to the study centre. Palivizumab was associated with a 49% relative reduction in the proportion of children with parental reported recurrent wheezing (three or more episodes of wheeze over one year) and a 51% relative reduction in physician diagnosed recurrent wheeze compared to the infants who had not received palivizumab (which included infants who had and had not been hospitalised for RSV infection) by two years (Simoes et al. 2007). When comparing the palivizumab treated group with the subgroup of those not receiving palivizumab who had not been hospitalised for RSV infection, the palivizumab group still had a 43% relative reduction in the proportion of children with parental reported recurrent wheezing and a 19% relative reduction in the proportion of children with physician diagnosed recurrent wheezing. It must be noted, however, that infants who had received palivizumab and had been hospitalised for RSV infection in the first year after birth were excluded from the study, therefore potentially biasing the results (Simoes et al. 2007).

1.10 Assessment of respiratory morbidity

1.10.1 Diary cards

Determining the frequency of respiratory symptoms such as cough and wheeze at follow-up can give an assessment of chronic respiratory morbidity. These symptoms can be documented by parent completed diary cards, which have been used to show prematurely born infants who develop RSV LRTI are more likely to cough and wheeze (Broughton et al. 2005) than those with no LRTI or RSV negative LRTIs and to assess infants' responses to medications for post-bronchiolitis wheeze (Fox et al. 1999; Bisgaard 2003).

1.10.2 Healthcare utilisation and costs of care

Respiratory morbidity can be assessed by healthcare utilisation and the associated healthcare related costs. This can be documented by calculating the number of GP attendances, hospital attendances (Accident and Emergency and out-patient), medication prescriptions and the number, duration and level of care (general ward, HDU or PICU) of hospital admissions. The associated costs can then be calculated from reference costing schemes (NHS reference costing scheme). Prematurely born infants with BPD who had been hospitalised for RSV infection (confirmed RSV by direct immunofluorescence) were shown to have had significantly more and longer hospital admissions to general paediatric wards, more admissions to PICU and more in-patient costs of care between their index hospital

admission (at a median age of seven months) and the end of the follow up period (two years of age) than the controls (Greenough et al. 2001). A further follow-up of those infants between the ages of two and four years showed the infants who had been hospitalised for RSV infection had more out-patient attendances and more medication prescriptions for both all medications and for respiratory medications (Greenough et al. 2004). They had greater healthcare costs for out-patient attendances, for all medication prescriptions and total cost of care over the follow-up period (Greenough et al. 2004). In addition, Sampalis demonstrated significant differences in infants born between 32 and 35 weeks of GA without BPD who had or had not had RSV infection (identified from hospital discharge codes, therefore included infants with 'probable' RSV infection) (Sampalis 2003). The RSV group had more and longer hospitalisations, more out-patient attendances and also a five-fold increase in mortality (8.1% versus 1.6%, p=0.001). All those studies included infants who had initially been hospitalised for RSV infection and as most infants who develop RSV are not hospitalised those studies may underestimate the healthcare burden. To address this Broughton et al prospectively followed infants born less than 32 weeks GA and documented RSV LRTIs both in hospital and in the community (Broughton et al. 2005). RSV was identified by either viral culture or direct immunofluorescence. The infants who developed RSV LRTIs had more and longer hospitalisations and more GP attendances than the infants who did not develop a LRTI, and longer hospitalisations, more PICU admissions and more GP attendances than the RSV negative LRTI group. Infants who developed RSV LRTIs not requiring hospitalisation required more GP

attendances than the no LRTI group (1.3 versus 0.4, p=0.049) and had significantly more wheeze in one month than both the no LRTI group (3.7 versus 0.6 days, p=0.005) and the RSV negative LRTI group (3.7 versus 0.7 days, p=0.004). It was also noted that the RSV negative group had more hospital admissions than the no LRTI group suggesting other viruses may cause chronic respiratory morbidity (Broughton et al. 2005). An aim of this thesis, therefore, was to investigate whether other viral LRTIs, as well as RSV LRTIs, regardless of whether hospitalisation was required, lead to increased healthcare utilisation and costs of care in prematurely born infants.

1.11 Assessment of lung function

1.11.1 Measurement of functional residual capacity

Functional residual capacity (FRC) can be measured by inert gas dilution techniques or by total body plethysmography. However, it is not possible to perform plethysmography as a bedside test on a NICU. Different methodological approaches have been described for the assessment of FRC by gas dilution, including the helium gas dilution technique (FRC_{He}) and the open-circuit, multiple-breath, inert-gas wash-in/out (FRC_{MBW}) technique (Hülskamp et al. 2006). Both approaches measure the volume of lung regions that communicate readily with the central airways during tidal breathing and therefore can be inaccurate when parts of the lung have long time constants. These techniques can be used prior to NICU discharge and at follow up and hence allows lung function to be tracked according to viral

status and to determine the impact of viral LRTIs on lung function. In this thesis both inert gas dilution techniques will be used to measure FRC in infants prior to NICU discharge and at follow up to allow lung function to be tracked. Total body plethysmography will be used to assess FRC at follow up (see section 1.11.4).

1.11.2 Ventilation Inhomogeneity

The multiple breath wash-in/out technique gives an assessment of ventilation inhomogeneity (VI), often measured as lung clearance index (LCI), a marker of small airway function. Infants with diseases including cystic fibrosis (Aurora et al. 2005) and congenital diaphragmatic hernia (Dotta et al. 2007) have been shown to have abnormally raised LCI. Viral LRTIs affect the small airways (Mok & Simpson 1982; Fjaerli et al. 2005) and thus an assessment of small airway function by LCI may demonstrate abnormalities not found by other lung function techniques and will be assessed in this thesis.

1.11.3 Compliance and resistance of the respiratory system by the single breath occlusion technique

Compliance (C_{rs}) and resistance (R_{rs}) of the respiratory system can be measured by the single breath occlusion technique. A brief airway occlusion at end inspiration induces a brief apnoea by the Hering-Breuer inflation reflex. The elastic recoil of the respiratory system can be measured by the pressure within a facemask. Relief of the obstruction results in passive

expiration, both flow and volume decrease exponentially once the flow has reached a peak, providing the mechanical characteristics of the lung remain unchanged. This allows the assessment of C_{rs} and the time constant (T_{rs}). R_{rs} can then be calculated from the formula $T_{rs}=C_{rs} \times R_{rs}$ (Milner & Rafferty 2003). This technique can be used prior to NICU discharge and at follow up and hence allows lung function to be tracked according to viral status and to determine the impact of viral LRTIs on lung function.

1.11.4 Plethysmographic measurement of FRC

During assessment by whole-body plethysmography, the subject is enclosed within a rigid, closed container and changes in alveolar pressure and volume are measured (Dubois et al. 1956a; Dubois et al. 1956b). FRC measured by plethysmography (FRC_{pleth}) allows the measurement of all gas within the thorax, which can then be compared to gas dilutional methods of measuring FRC to give an estimation of gas trapping within the thorax (e.g. the FRC_{He}:FRC_{pleth} ratio). Broughton et al in a study of infants born less than 32 weeks of gestation measured at one year corrected age, found a mean FRC_{pleth} of 30 ml/kg and a mean FRC_{He}:FRC_{pleth} ratio of 0.82 (Broughton et al. 2007).

1.11.5 Plethysmographic measurement of airway resistance

Whole-body plethysmography also allows measurement of airway resistance (R_{aw}). Plethysmographic resistance measurements are performed at

standard BTPS conditions, using a heated rebreathing bag, so that any changes in the flow or pressure recordings reflect changes in R_{aw} rather than changes in humidity and temperature of the respired gas throughout the respiratory cycle (Gappa et al. 2006). New methods of whole-body plethysmography do not measure under standard BTPS conditions and use electronic or mathematical algorithms to compensate for thermal artefacts. Unfortunately, the current algorithms are not sophisticated enough to achieve reliable measurements in infants and very young children and thus may not be appropriate for use in this population (Broughton et al. 2007). For the follow up measurements in this study whole-body plethysmography measured under standard BTPS conditions was used.

1.11.6 The rapid thoracoabdominal compression technique

The rapid thoracoabdominal compression technique (RTC) generates flow-volume curves in infants. It can be carried out using the tidal RTC method or the raised volume RTC method (Lum et al. 2006). Both involve wrapping an inflatable jacket around a sedated infant's chest and abdomen and allowing the infant to breathe through a facemask and pneumotachograph. In the tidal RTC technique at least five regular tidal breaths are obtained and then an initial jacket pressure of 20–30 cm H_2O is applied usually at end-inspiration, which applies a pressure around the chest and abdomen to force expiration (Lum et al. 2006). The manoeuvre is repeated with increasing jacket pressure until further increases do not elicit further increases in flow at functional residual capacity (FRC) (Lum et al. 2006). From this, maximal flow

at FRC (V_{max}FRC) is reported. With the raised volume RTC technique the infant's lungs are initially inflated to a preset pressure prior to RTC, to force expiration from a volume beyond tidal range and then the same methodology as for the tidal RTC technique is applied. Results reported from the raised volume RTC technique include forced vital capacity (FVC), forced expired volume in 0.5 sec ($FEV_{0.5}$), forced expiratory flow when 50% of FVC has been expired (FEF_{50}), forced expiratory flow when 75% of FVC has been expired (FEF_{75}) and FEF when 25–75% of FVC has been expired ($FEF_{25–75}$) (Lum et al. 2006). This, however, is an invasive technique and potential problems include gastrointestinal problems or pneumothorax, particularly in infants with inhomogeneous lung disease such as prematurely born infants with BPD or infants with respiratory insufficiency.

1.12 Premorbid lung function and morbidity associated with viral LRTI

Infants who become symptomatic with RSV infection may do so because of a functional predisposition.

1.12.1 Previously healthy, term born infants

The Tucson Children's Respiratory Study (Martinez et al. 1988) assessed lung function, V_{max}FRC, FRC, conductance and tidal breathing parameters, at eight weeks of age and then prospectively followed the infants until one year of age. Although there were no significant differences in lung function between infants who did and did not develop LRTIs, it was demonstrated that

infants who developed a wheezy LRTI had a reduced conductance of the respiratory system compared to those infants who did not develop a LRTI (0.028 versus 0.035 L/sec/cmH_2O, $p<0.05$) (Martinez et al. 1988). No virological results, however, were reported. Those results could be interpreted as indicating that diminished lung function predicts those who wheeze with a LRTI rather than those predisposed per se to a viral LRTI. Another prospective study measuring lung function before and after bronchiolitis at one year of age found that infants with evidence of reduced pre-existing respiratory function (lower V_{max}FRC) and lower respiratory tract symptoms prior to infection, had a non significant ($p<0.06$) trend to develop bronchiolitis (Young et al. 1995). Virology results, however, were only available for the two infants who were admitted to hospital, both of whom were RSV positive; the other infants who were not admitted had a doctor diagnosis of bronchiolitis. A follow-up study (Turner et al. 2002) of that cohort (Young et al. 1995) highlighted that the infants with bronchiolitis had reduced lung function (%FEF_{25-75}) and at a similar level to premorbid levels, as assessed by z-scores, at 11 years of age. That study (Turner et al. 2002) suggests viral LRTIs affect infants predisposed to poor lung function rather than resulting in it and thus preventing viral LRTIs may not result in reduced chronic respiratory morbidity in those infants.

1.12.2 Prematurely born infants

In a prospectively followed cohort, infants born prior to 32 weeks of gestation and developing RSV LRTI, 40% of whom required hospitalisation, in infancy

had an increased R_{rs} before discharge from the neonatal unit when compared with infants not developing a RSV LRTI (Broughton et al. 2006). The control group consisted of infants who did not develop a LRTI and infants who developed a LRTI caused by another virus. Thus an aim of this thesis was to assess the impact of premorbid lung function on the development of RSV and other viral LRTIs in prematurely born infants.

Prematurely born infants have lung function abnormalities as a result of antenatal and postnatal insults (Chess et al. 2006). Therefore, in this thesis lung function was assessed prior to NICU discharge and was related to the development of subsequent viral LRTIs to help to predict which infants will go on to develop RSV or other viral LRTIs.

1.13 Genetic predisposition to RSV LRTI

1.13.1 Single nucleotide polymorphisms associated with RSV LRTI

Single nucleotide polymorphisms (SNPs) in several genes coding for cytokines associated with RSV infection have been associated with an increased risk of developing RSV bronchiolitis requiring hospitalisation (Table 1.4). Most of these studies have investigated very homogenous ethnic populations, have been retrospective in nature and have involved only term born infants or small numbers of prematurely born infants grouped together with term born infants. In addition, most did not have a similar control group (i.e. results of the index patients were compared to healthy adults or blood

donors) and did not use the sensitive method of PCR to identify cases. One prospective study has compared infants with RSV infection requiring hospitalisation to those with RSV infection not requiring hospitalisation and a control group (healthy adults) (Tal et al. 2004). Two SNPs in TLR-4 were compared and it was reported that infants hospitalised for RSV LRTI had a common TLR-4 mutation whereas those with RSV infection not requiring hospitalisation and the control group did not, suggesting this SNP is associated with severe RSV LRTI rather than RSV infection per se .

SNPs were chosen for investigation in this study as they had previously been associated with an increased risk of severe RSV infection in infants born at term (Hoebee et al. 2004; Wilson et al. 2005; Janssen et al. 2007; Helminen et al. 2008) or born prematurely (Siezen et al. 2009), an increased risk of developing RDS (Lahti et al. 2004), a decreased risk of developing BPD (Hadchouel et al. 2008), an increased risk of recurrent wheeze at one year of age after RSV LRTI (Ermers et al. 2011) or reduced preschool lung function (Simpson et al. 2005).

An aim of this thesis was to assess whether prematurely born infants had a genetic predisposition to developing a RSV LRTI.

Table 1.4: Studies investigating SNPs associated with severe RSV infection requiring hospitalisation, in previously healthy, term-born infants

Reference	Cases (n)	Controls	Control group (n)	Number of SNPs tested	SNPs associated with RSV
(Hull, 2000)	117 (10 preterm)	Cord blood donors	180	1	IL-8
(Löfgren et al. 2002)	86	Healthy neonates	95	12	SP-A1, SP-A2
(Lahti et al. 2002)	84	Healthy aged matched infants	93	9	SP-D
(Choi et al. 2002)	105	Healthy blood donors	315	10	IL-4
(Hoebee et al. 2003)	200	Healthy adults	447	3	IL-4, IL-4Ra
(Hull et al. 2003)	580 (116 preterm)	Healthy neonates	580	5	CCR5
(Gentile 2003)	77	None	0	5	IFN gamma, IL-6, IL-10, TGFB1
(Hoebee et al. 2004)	204	Healthy adults	447	3	IL-10
(Hull et al. 2004)	580 (116 preterm)	Healthy neonates	580	63	IL-8, RASSF6
(Wilson et al. 2005)	580 (116 preterm)	Healthy neonates	580	8	IL-10
(Krueger et al. 2006)	154	Healthy adults	270	5	Nil
(Puthothu et al. 2006)	131	Adults (either healthy or with asthma)	323 asthma, 270 healthy	5	IL-8 (compared to asthma group, not healthy group)
(Puthothu et al. 2006)	131	Healthy adults	270	4	IL-13
(Puthothu et al. 2007)	154	Healthy adults	270	6	IL-18
(Janssen et al. 2007)	470 (113 preterm)	Healthy adults	447	384	VDR, JUN, IFNA5, NOS2
(Fjaerli et al. 2007)	18	Infants with RSV not requiring hospitalisation	5	15	FAM102A, TNFRSF25, STMN3
(Amanatidou et al. 2008)	106	Healthy adults	120	7	RANTES
(Mailaparambil et al. 2008)	156	Healthy adults	270	19	TLR-9,10
(Hashimoto et al. 2008)	81	Healthy adults	98	9	PGIS
(Mailaparambil et al. 2008)	156	Healthy children	296	14	Nil

1.13.2 SNPs associated with wheezing post-RSV LRTI

A SNP in the gene coding for IL-8 (Goetghebuer et al. 2004) and another in the gene coding for IL-13 (Ermers et al. 2007) have been shown to be associated with post-RSV bronchiolitis wheeze at six years of age. In the study by Ermers et al, the IL-13 SNP was not associated with wheezing in the first three years post-RSV bronchiolitis, but was associated with wheezing at six years (Ermers et al. 2007). Those results suggest that early and late wheezing after RSV bronchiolitis may result from a different genetic background.

A further aim of this thesis was to assess whether prematurely born infants have a genetic predisposition to develop subsequent wheeze and chronic respiratory morbidity after developing a RSV LRTI.

1.13.3 SNPs associated with other viral LRTIs in infancy

One study has investigated the genetic predisposition to respiratory viruses other than RSV. Previously healthy term-born infants less than six months of age, who had been hospitalised for non-RSV bronchiolitis, and especially HRV bronchiolitis, were found to have a polymorphism in the gene coding for IL-10 (homozygosity for A at -1082) (Helminen et al. 2008). The same SNP had been associated with RSV bronchiolitis requiring mechanical ventilation (Wilson et al. 2005), although no association with RSV was found in that study (Helminen et al. 2008).

1.14 Summary

RSV LRTI has been associated with chronic respiratory morbidity including cough, wheeze, a diagnosis of asthma, lung function abnormalities and increased healthcare utilisation and cost of care in both term and prematurely born infants. Other respiratory viruses may cause an acute clinical picture similar to that of RSV infection. Few data exist, however, on their affect on subsequent respiratory morbidity, especially in prematurely born infants.

Term born and prematurely born infants may be predisposed to develop RSV LRTI by reduced premorbid lung function. Whether, however, reduced premorbid lung function predisposes prematurely born infants to other viral LRTIs has rarely been investigated. In addition, term born infants may be genetically predisposed to develop RSV LRTI, but whether prematurely born infants are similarly predisposed has not been investigated.

1.15 Hypotheses

- RSV and other viral LRTIs in prematurely born infants will result in increased healthcare utilisation and cost of care.
- RSV and other viral LRTIs in prematurely born infants will result in reduced lung function and increased respiratory symptoms at follow-up.
- Reduced premorbid lung function will predispose prematurely born infants to RSV and other viral LRTIs.
- Host genotype may influence the likelihood of developing a symptomatic RSV LRTI in prematurely born infants.

1.16 Aims

- To prospectively follow a cohort of prematurely born infants until two years corrected age, documenting all of their LRTIs, both in hospital and in the community, and to assess chronic respiratory morbidity (respiratory symptoms, lung function, healthcare utilisation and cost of care) compared to controls.
- To determine whether RSV and other viral LRTIs result in a similar outcome compared to infants without viral LRTIs.
- To assess whether diminished lung function prior to neonatal discharge predisposes prematurely born infants to symptomatic RSV and other viral LRTIs.
- To determine whether prematurely born infants have a genetic predisposition to RSV LRTI and chronic respiratory morbidity.

Chapter 2: Methods

2.1 Protocol

Infants born prior to 36 weeks of gestation were eligible for entry into the study when they reached 36 weeks postmenstrual age (PMA) in the six months prior to the RSV season (October 1st to March 31st) (Clark, 2000) in 2008 or 2009. After discharge, infants were prospectively followed; those born in 2008 were followed until two years corrected age (2008 cohort), those born in 2009 until one year corrected age (2009 cohort). All recruited infants were exposed to at least one RSV season on neonatal/maternity unit discharge. Parents of infants were approached on either the postnatal ward or the neonatal unit. Infants with congenital or chromosomal abnormalities were excluded from the study, as were infants who lived too far from the hospital to make home visits feasible.

At 36 weeks PMA and prior to neonatal/maternity unit discharge, infants had lung function measured: lung volume using a helium gas dilution technique, C_{rs} and R_{rs} using the single breath occlusion technique and lung volume and ventilation inhomogeneity (lung clearance index) by the multiple breath wash-in/out technique. In addition, blood or buccal swabs were obtained from infants for DNA extraction and SNP analysis.

All LRTIs were documented from discharge until one (both cohorts) or two (2008 cohort only) years corrected age.

Following neonatal unit discharge, parents were asked to contact the research team whenever their infant had a LRTI and were telephoned every two weeks by a researcher. A researcher visited the home or hospital on every occasion an infant had a LRTI and obtained a NPA. NPAs were analysed by real time PCR for 13 respiratory viruses.

An infant was considered to have a viral LRTI if they had coryzal symptoms with or without fever and either a raised respiratory rate for their age (>60 for infants), crackles or wheeze on chest auscultation or respiratory distress (e.g. subcostal recession). This included infants with diagnoses including bronchiolitis, pneumonia and viral induced wheeze. The severity of the LRTI was assessed by the need for hospitalisation- either 'severe' warranting hospitalisation or 'non-severe' not requiring hospitalisation.

When the infant was 11 (both cohorts) and 23 (2008 cohort only) months corrected age, parents were asked to complete a diary card (Appendix 1) for one month. Parents recorded on a daily basis if their infant coughed, wheezed, visited a medical practitioner and/or required respiratory related medication. In addition, parents completed a respiratory health-related questionnaire (Appendix 2) at one (both cohorts) or two (2008 cohort only) years corrected age.

Lung function was measured at one year corrected age. Measurements included those carried out at 36 weeks PMA and in addition whole body

plethysmography was performed to measure lung volume and airways resistance.

Healthcare utilisation and cost of care were calculated at one (both cohorts) and two (2008 cohort only) years corrected age.

The study was approved by the Research Ethics Committee of King's College Hospital NHS Trust.

2.2 Anthropometric measurements

2.2.1 Weight

Weight was measured at 36 weeks PMA and at one year corrected age using a digital scale (Marsden digital baby/toddler scale, Marsden weighing group, Oxfordshire, UK). Before weighing the infant, the scale was zeroed, the infant was then placed naked on the scale and the weight in kilograms recorded. The accuracy of the weighing scales was determined using weights of one, two, three and four kg (i.e. the expected weights of infants at 36 weeks PMA) and weights of 6, 10 and 14 kg (i.e. those expected at one year corrected age). The assessment was performed in triplicate. For the 1-4 kg weights the mean (range) difference between the actual and measured weights was 2 (0-3) g and for the 6-14 kg weights the mean (range) difference was 19 (10-35) g.

2.2.2 Length

Length was measured at one year corrected age using an infant stadiometer (Holtain Ltd, Crymych, UK). The accuracy of the stadiometer was checked using 60 cm and 90 cm metal calibration rods. The assessment was performed in triplicate. The mean difference between rod length and measured length was 0.07 cm (0.1 %) at 60 cm and 0.03 cm (0.04 %) at 90 cm.

2.3 Measurement of lung function at 36 weeks PMA

2.3.1 FRC measured by the helium gas dilution technique

2.3.1.1 Equipment

Lung volume was assessed using a commercially available helium gas dilution system (EBS 2615, Equilibrated Bio Systems, New York, USA). The FRC system contained a 500 mL re-breathing bag, the system reservoir, enclosed in an airtight perspex box. The re-breathing bag, containing a mixture of helium (approximately 10%) and oxygen, was connected to a three-way valve to which a facemask could be attached. The helium analyser was calibrated using a certified calibration gas prior to each test. Release of the valve from its standby position open to atmosphere resulted in connection of the patient port to the re-breathing bag. The helium analyser was contained in a separate unit and connected to the re-breathing bag via flexible tubing. The gas in the re-breathing bag was circulated continuously

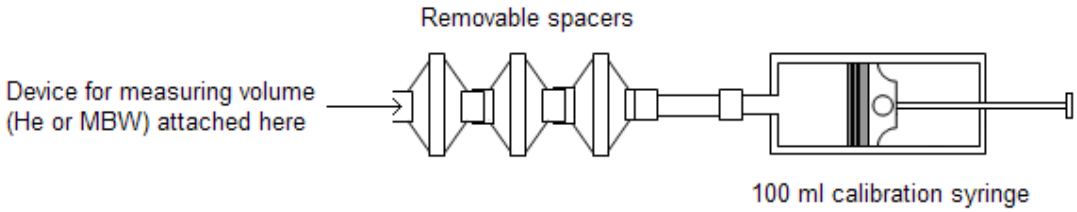
through the analyser during the measurement and helium concentration in the circuit was displayed in real time on a LCD monitor. Helium equilibration was deemed to have occurred if there was no change in helium concentration over a 15 second period. The initial and equilibration helium concentrations were used to calculate FRC.

2.3.1.2 *In vitro* assessment

The accuracy of the measurement of volume by the helium gas dilution technique was assessed using a lung model which consisted of a 100 mL certified calibration syringe (5510, Hans-Rudolph Inc, Montana, USA), with a stroke volume of 20-100 mL delivered at a rate of 30 strokes per minute, attached to identical spacers to vary the volume of the model (Figure 2.1).

Figure 2.1: A schematic diagram showing the lung model

Adapted from PhD thesis by S. Broughton, King's College London, 2009.



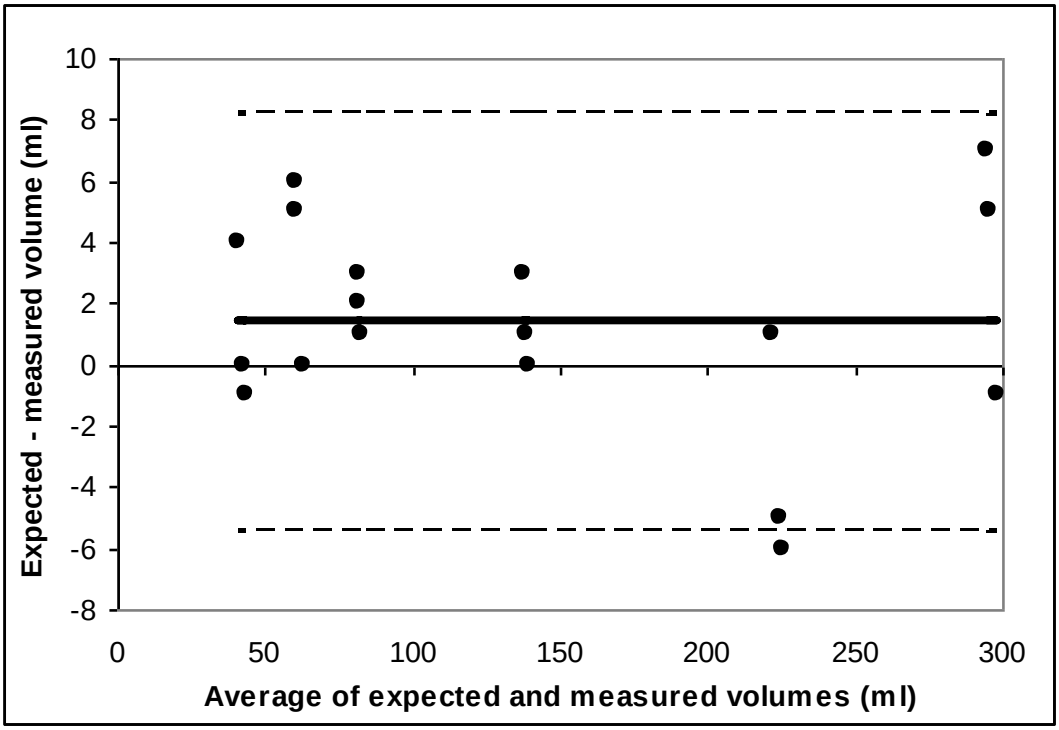
This was then attached to the patient port of the three way valve of the helium gas analyser using a connector. The volumes of the lung models were determined by water displacement. During the measurement, equilibration was deemed to have occurred when there had been no change

in the helium concentration over a 15 second period. FRC was measured three times at each volume tested.

The volumes used in the model were chosen to encompass those likely to be measured. To represent neonates, assuming bodyweights of 2.0–3.5 kg with predicted FRC in the range of 40-90 mL, volumes of 43, 63, and 83 mL were used and to represent infants at one year corrected age with likely bodyweights of 8-12 kg, and predicted FRC ranging from 160-300 mL, volumes of 139, 222 and 298 mL were used. The mean (SD) difference in the expected and measured volumes was 1.0 (3.4) mL, which is a smaller volume than would be clinically meaningful, demonstrating the helium gas dilution technique accurately measured the volumes of the lung models (Figure 2.2).

Figure 2.2: Bland and Altman plot of expected and measured volumes (mL) using the helium gas dilution technique

The solid line is the mean value and the dotted lines ± 1.96 standard deviations.



2.3.1.3 *In vivo* assessment

The sleeping infant lay supine, with their head in the neutral position. A facemask (Rendell Baker, Laerdal, Norway) was held snugly over the mouth and nose; silicone putty (Promedics, Port Glasgow, UK) was placed around the mask to achieve an airtight seal. The facemask was connected to the re-breathing bag via a three-way valve. The three-way valve was switched at the end of expiration, so that the infant subsequently breathed from the re-breathing bag. The initial and equilibration helium concentrations were used

to calculate FRC and this was corrected for oxygen consumption assumed to be 7 mL/kg/min (Hey, 1969) and body temperature and pressure, saturated (BTPS) conditions. FRC was measured at least twice in each infant, results of two measurements were considered acceptable if they were within ten per cent of each other. The reproducibility of FRC was tested on nine infants with two sets of tests carried out on the same day. These results demonstrated good short term reproducibility with a co-efficient of variation percentage (standard deviation) of 1.9% (1.7) and a mean difference of 0.9 mL.

2.3.2 FRC measured by the multiple breath wash-in/out technique

2.3.2.1 Equipment

The open circuit multiple breath wash-in/out system (MBW) (Exhalyzer D, Ecomedics, Duernten, Switzerland) included an ultrasonic flowmeter (USFM) with a deadspace reducer (reducing the internal dead space of the flowmeter to 1.2 mL) and disposable bacterial filter (Spirette, EcoMedics AG, Duernten, Switzerland) inserted into it, connected to a bias flow (8-12 L/min). The USFM and bias flow were both connected to the control unit and switch box which controlled the switching between air and the tracer gas mix. The control unit and switch box were also connected to a laptop computer (Dell, UK) running WBreath software (WBreath version 3.19.8.0, ndd Medizintechnik AG, Zurich, Switzerland). The USFM measured the inert tracer gas (approximately 5% sulphur hexafluoride [SF6] mixed with 21%

oxygen, and balance Nitrogen) continuously near the airway opening together with the associated flow of respired gas mixture. The USFM allows measurement of molar mass (MM) in the range of 20-45 g/mol with a precision of 0.01 g/mol. Inspiratory and expiratory flows were integrated to calculate inspired and expired gas volumes (Schibler & Henning 2001; Schibler et al. 2002; Pillow et al. 2004). As MM influences the transit time of the ultrasonic signal, inert gases with a MM sufficiently different from normal respired gas can be detected and used as a tracer gas for the MBW technique.

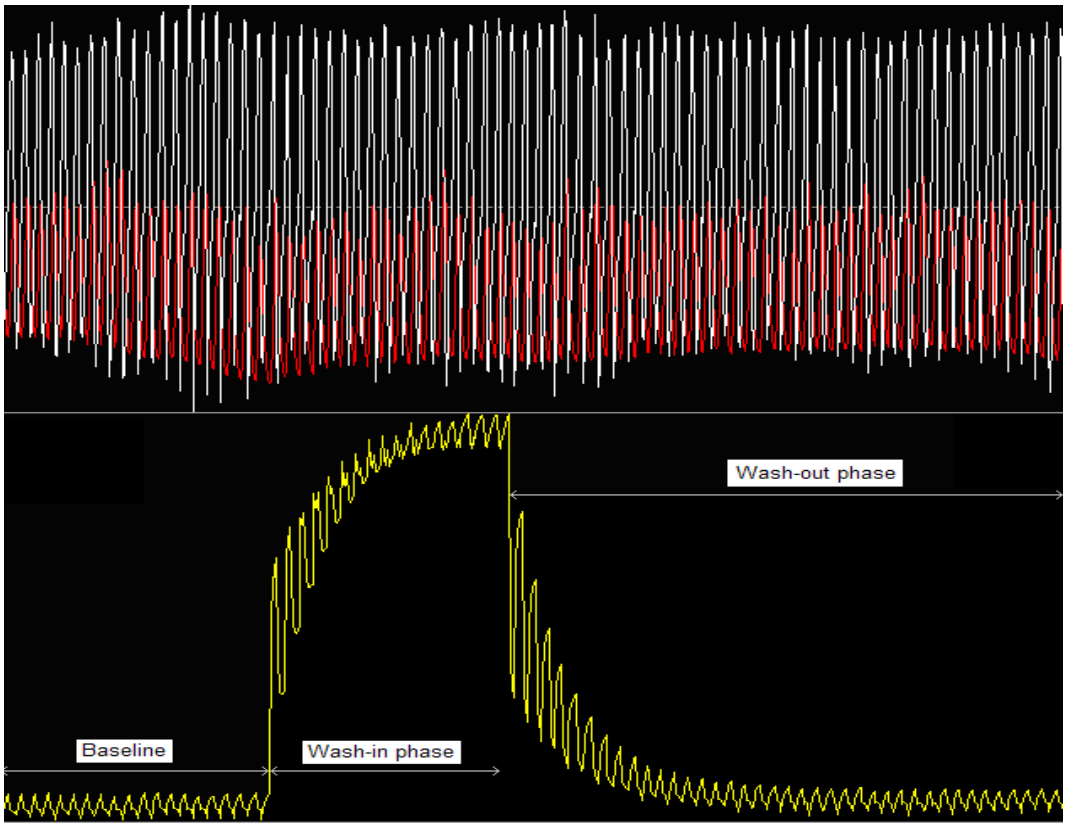
Calibration was performed before each test. Room temperature and humidity were measured with a humidity and temperature meter (RS 1360, RS Components Ltd, Northants, UK) and were entered into the computer programme. Volume was then calibrated using a 100 mL certified calibration syringe (5510, Hans-Rudolph Inc, Montana, USA).

After a period of baseline tidal volume measurements in air, the “wash-in” phase commenced and the bias flow was automatically switched to the tracer gas by the computer software. “Wash-in” was maintained until the change in end expiratory molar mass varied by less than 0.01 g/mol over three consecutive breaths. The “wash-out” phase was then automatically commenced by the computer software, and the bias flow was switched to air again. “Wash-out” continued until the change in MM visibly returned to baseline (Figure 2.3). Data was saved in the “raw” format and analysis of the

data was carried out offline. The MBW technique is also able to measure ventilation inhomogeneity (VI), measured as lung clearance index (LCI).

Figure 2.3: The MBW technique output

The white line is the flow trace, the red line is the volume trace, and the yellow line is the molar mass trace.



2.3.2.2 *In vitro* assessment

The accuracy of the measurement of volume by the MBW technique was assessed using the same lung model and the same volumes as for the helium gas dilution technique (section 2.3.1.2). The USFM was attached to the lung model and the data from each test were saved in the "raw" format, i.e. prior to correction for any factors such as temperature or side chamber reservoir effect (see below). "Wash-in" measures from a baseline of no

tracer gas in the lung to the point of tracer gas equilibration within the lung (three consecutive breathes with a change in MM of less than 0.01 g/mol, automatically calculated by the computer software) (Figure 2.3). Conversely, “wash-out” measures from the point of tracer gas equilibration in the lung to the point of equilibration of removal of all tracer gas from the lung, as determined by the operator viewing the trace and seeing the MM trace revert back to baseline (Figure 2.3).

The MM of the gas in the main stream of the flowmeter was computed from the transit time which was directly proportional to the density of the medium, which itself was dependent on the precise temperature along the sound transmission path. Because minor changes in temperature along the sound transmission path are present during tidal breathing, the changes in temperature along the sound transmission path must be simulated using a temperature model. The temperature model uses the measured flow, USFM dead space and the preset temperature of the inspiratory and expiratory gas to calculate the temperature changes along the sound transmission path (Hülskamp et al. 2009). The effects of changes in temperature on MM were accounted for using the temperature model suggested by Latzin et al (2007).

In the lung model, the analysis also included correction for a side chamber reservoir effect (“step response”), but not body temperature and pressure, saturated (BTPS) conditions. FRC was determined by calculating the sum of the product of tidal volume and the end expiratory molar mass change for each breath of the “wash-in”. The volume of inhaled or exhaled tracer gas

was automatically corrected for re-inspired gas between the bias flow and the sensor “mid-point” and for the deadspace between the sensor “mid-point” and the mask (Hülskamp et al. 2009). LCI was calculated as the number of volume turnovers (cumulative inspired or expired volume divided by FRC) required to reduce the end-tidal tracer gas concentration to 1/40th of the concentration at the start of “wash-out” (Schibler et al. 2002) (or if using the “wash-in”; increase to 39/40th).

Three technically acceptable “wash-in” and “wash-out” traces were obtained for each lung model. Analysis demonstrated measurement of the “wash-in” phase to be more accurate than the “wash-out” phase which always underestimated the volume (Table 2.1 and Figures 2.4 and 2.5), thus for this thesis the results from the “wash-in” phase analysis were used.

Table 2.1: *In vitro* results of FRC measured by the MBW technique

Test volume (mL)	Measured volume (Wash-in) (mL)	Measured volume (Wash-out) (mL)
43	51.7	35.4
43	49.9	33.8
43	51.8	36.1
63	60.5	47.4
63	68.5	51.4
63	76.7	49.3
83	78.9	67.3
83	77.7	65.5
83	84.7	66.9
139	143.1	130.4
139	144.0	131.7
139	130.1	132.9
222	195.9	219.1
222	199.0	219.4
222	195.7	220.0
298	275.5	288.9
298	251.3	264.8
298	253.6	280.0

Figure 2.4 Bland and Altman plot of expected and measured volumes (mL) analysing the "wash-in" phase of the MBW technique

The solid line is the mean value and the dotted lines ± 1.96 standard deviations.

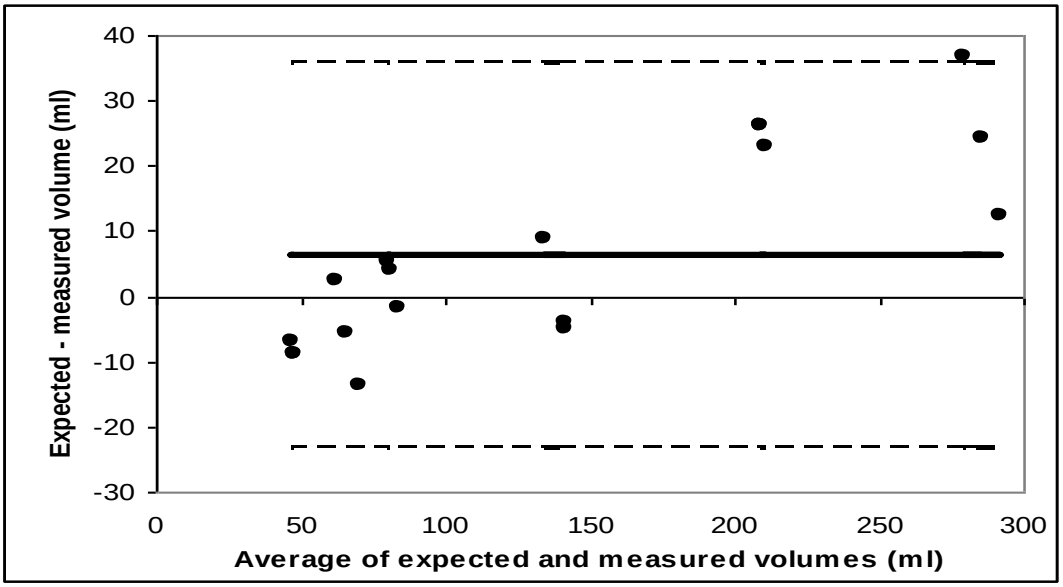
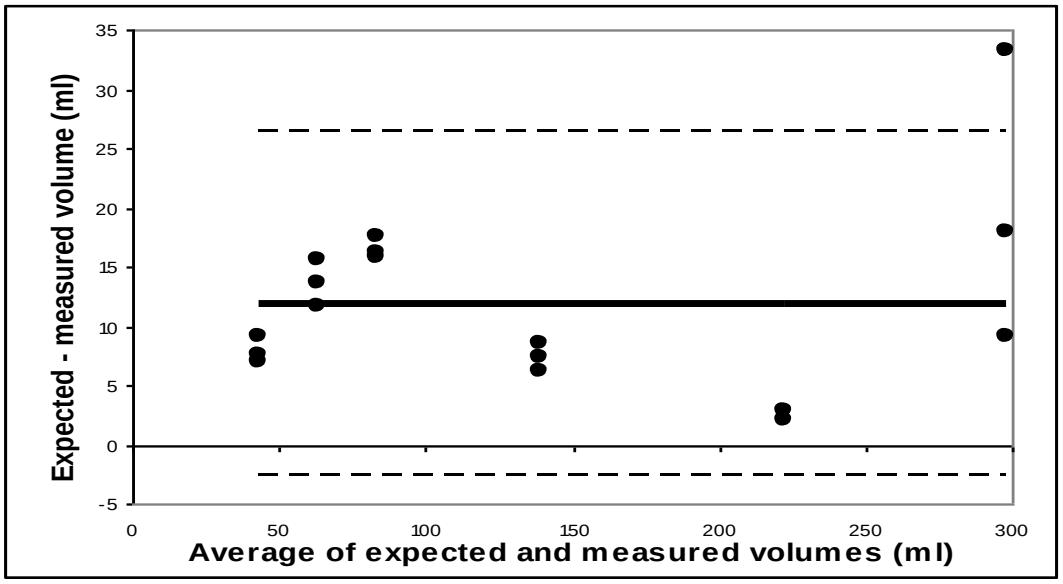


Figure 2.5: Bland and Altman plot of expected and measured volumes (mL) analysing the "wash-out" phase of the MBW technique

The solid line is the mean value and the dotted lines ± 1.96 standard deviations.



2.3.2.3 *In vivo* assessment

The infant lay supine, with their head in the neutral position and a facemask (Rendell Baker, Laerdal Norway) held snugly over their mouth and nose; silicone putty (Promedics, Port Glasgow, UK) was placed around the mask to achieve an airtight seal. After a period of baseline tidal breathing, breathing in air, the "wash-in" phase commenced and the tracer gas was inspired. "Wash-in" was maintained until the change in end expiratory molar mass varied by less than 0.01 g/mol over three consecutive breaths. The "wash-out" phase was then automatically commenced and the infant began breathing air again. "Wash-out" continued until the change in MM returned to baseline (Figure 2.3). FRC and LCI were determined as for the lung model (section 2.3.2.2) but analysis also corrected for BTPS and the "effective" dead space of the mask used (4 mL at 36 weeks PMA and 13 mL at one year corrected age), which was subtracted from the USFM "mid-sensor" FRC value to obtain the infant's FRC.

Data were accepted for analysis if obtained during regular breathing, there was no evidence of leak, i.e. no sudden change in MM or flow and there were no sighs in the five breaths immediately prior to or the ten breaths immediately following commencement of the wash-in or wash-out (Hülskamp et al. 2009).

The reproducibility of FRC_{MBW} and LCI was tested on ten infants with two sets of tests carried out on the same day. These results demonstrated good

short term reproducibility with a co-efficient of variation percentage (standard deviation) of 4.8% (3.8) and 4.6% (4.5) respectively and a mean difference of 8.3 mL and 0.1 respectively.

2.3.3 Compliance and resistance of the respiratory system

The single breath occlusion technique was used to non-invasively determine total respiratory system compliance (C_{rs}) and resistance (R_{rs}) (LeSoeuf et al. 1984). The technique involves briefly occluding the airway at end inspiration which results in a brief apnoea and invokes the Hering-Breuer inflation reflex (HBIR). After the occlusion is terminated passive expiration occurs, i.e. there is no expiratory respiratory muscle activity. The time constant (T_{rs}), the time for 63% of the tidal volume to leave the lung, can then be measured from the slope of the plot of expiratory volume against expiratory flow (Milner & Rafferty 2003). C_{rs} is calculated from the formula: change in volume/change in pressure, measured after extrapolating the flow volume slope to zero. R_{rs} is calculated from the formula: $T_{rs} = C_{rs} \times R_{rs}$.

2.3.3.1 Equipment

A pneumotachograph (Mercury F10L; GM Engineering, Kilwinning, UK) attached to a differential pressure transducer (range: ± 0.2 kPa, MP 45, Validyne engineering, California, USA) was used to measure airflow. Airway pressure was measured from a side-port on the pneumotachograph using a differential pressure transducer (range: ± 10 kPa, MP 45, Validyne

engineering, California, USA). The signals from the transducers were amplified (Validyne CD280, Validyne engineering, California, USA) and acquired, analysed and displayed in real time on a computer (Dell, UK) running a custom made software program written using Labwindows software (National Instruments, Texas, USA) with analogue-to-digital sampling at 100 Hz (DAQCard 16XE-50, National Instruments, Texas, USA). Flow was digitally integrated by the computer software to give volume.

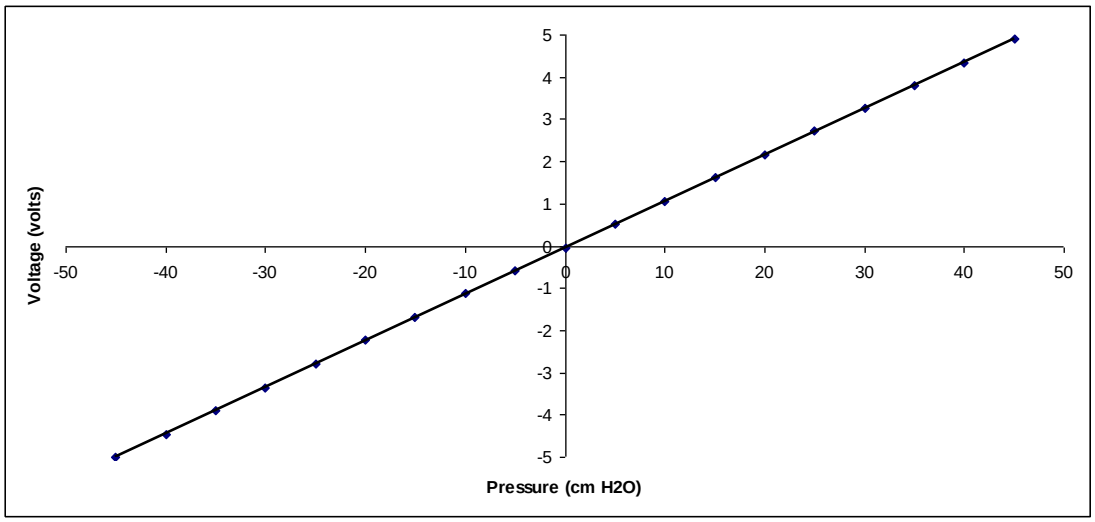
2.3.3.2 Calibration

Prior to each measurement, airway pressure was calibrated using a digital pressure meter (Comark C9505IS, Welwyn Garden City, UK) and flow through the pneumotachograph was calibrated using a flow meter (0-12 L/min, Platon, Roxspur M&C Ltd, Hants, UK).

2.3.3.3 Validation of transducer linearity

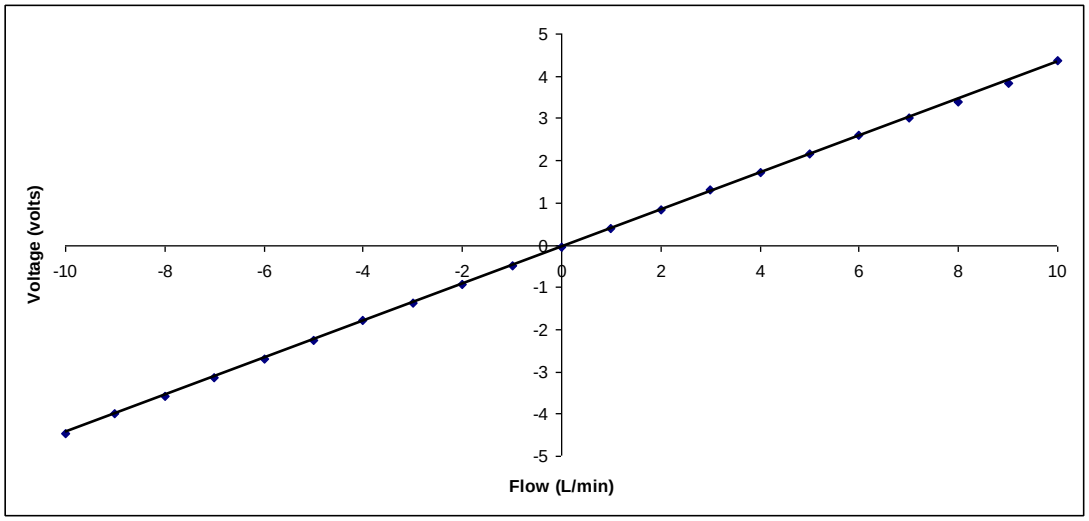
The airway pressure transducer was assessed for linearity over the range of ± 45 cmH_2O using a digital pressure meter (Comark C9505IS, Welwyn Garden City, UK). Applied pressure was compared to the voltage output from the amplifier using BioBench software (version 1.0, National Instruments, Texas, USA). The airway pressure transducer had a linear response (r^2 >0.99) (Figure 2.6).

Figure 2.6: Plot of pressure against voltage produced by the airway pressure transducer



The linearity of the flow transducer and associated pneumotachograph were measured using a flow meter (Platon, 0-12L, Roxspur M&C Ltd, Hants, UK) attached to the pneumotachograph. Flow was passed through the pneumotachograph in one litre per minute increments across the range 10 L/min in both directions (inspiratory and expiratory) and the results plotted against the flow delivered by the flow meter. The pneumotachograph had a linear response (r^2 >0.99) (Figure 2.7).

Figure 2.7: Plot of flow against voltage produced by the pneumotachograph and associated flow transducer



2.3.3.4 Assessment of the frequency response

The frequency response of the airway pressure transducer was assessed by measuring the response of the system to a quasi-instantaneous drop in pressure by bursting a balloon attached to the end of the pneumotachograph. The response time (T_r) (the time taken for pressure to fall from 90% to 10%) was 28 milliseconds. The frequency response was calculated from the equation:

Frequency response =1/3*Tr*

and was 11.9 Hz.

The frequency response of the flow transducer and pneumotachograph was assessed by measuring the response of the system to a quasi-instantaneous drop in pressure by bursting a slowly deflating balloon (which therefore gave

a constant flow) attached to the end of the pneumotachograph. The response time was 30 milliseconds. The frequency response was calculated as above and was 11.1 Hz.

2.3.3.5 *In vivo* assessment

A facemask (Rendell Baker, Laerdal, Norway) connected to the pneumotachograph was placed over the mouth and nose of the sleeping infant. A rim of silicone putty (Promedics, Port Glasgow, UK) was placed around the edge of the facemask to ensure a good seal. End inspiratory airway occlusions were performed by manually occluding the distal end of the pneumotachograph. End inspiration was identified from the flow signal. Only occlusions during which there was no flow, a mouth pressure plateau of at least 100 milliseconds and a linear flow-volume plot following the occlusion were considered acceptable (Gappa et al. 2001).

Briefly occluding the airway and maintaining the lung volume above end expiratory volume, induced the Hering-Breuer inflation reflex (HBIR) and passive expiration occurred when the occlusion was released (Figure 2.8). The resulting relaxed expiratory flow volume curve and the pressure time trace were analysed to calculate the expiratory time constant (T_{rs}), C_{rs} and R_{rs}. A linear segment in the decelerating flow volume curve was selected, and a least squares best fit line was drawn and extended to cross the volume axis at zero flow to correct for any dynamic elevation of FRC. The T_{rs} was measured from the slope of the best fit line on the expiratory flow volume

curve. C_{rs} was calculated from the extrapolated expired volume intercept, divided by the change in airway pressure; i.e. difference between plateau pressure during the occlusion and end expiratory pressure (zero). R_{rs} was calculated by rearranging the formula: $T_{rs} = C_{rs} \times R_{rs}$. The flow volume curve was analysed using the computer software and only those with a linearity of $\geq$0.99 for at least 40% of the curve were used (Figure 2.9). Eight to ten occlusions were recorded and the mean value calculated. At least ten breaths were allowed between occlusions. The resistance of the apparatus was known and was subtracted from the result.

The reproducibility of R_{rs} and C_{rs} was tested on six infants with two sets of tests carried out on the same day. These results demonstrated good short term reproducibility with a co-efficient of variation percentage (standard deviation) of 9.1% (2.9) and 10.7% (3.7) respectively and a mean difference of 0.1 $cmH_2O/L/s$ and 0.2 ml/cmH_2O respectively.

Figure 2.8: Diagram showing an occlusion at end inspiration and the resulting Hering-Breuer inflation reflex

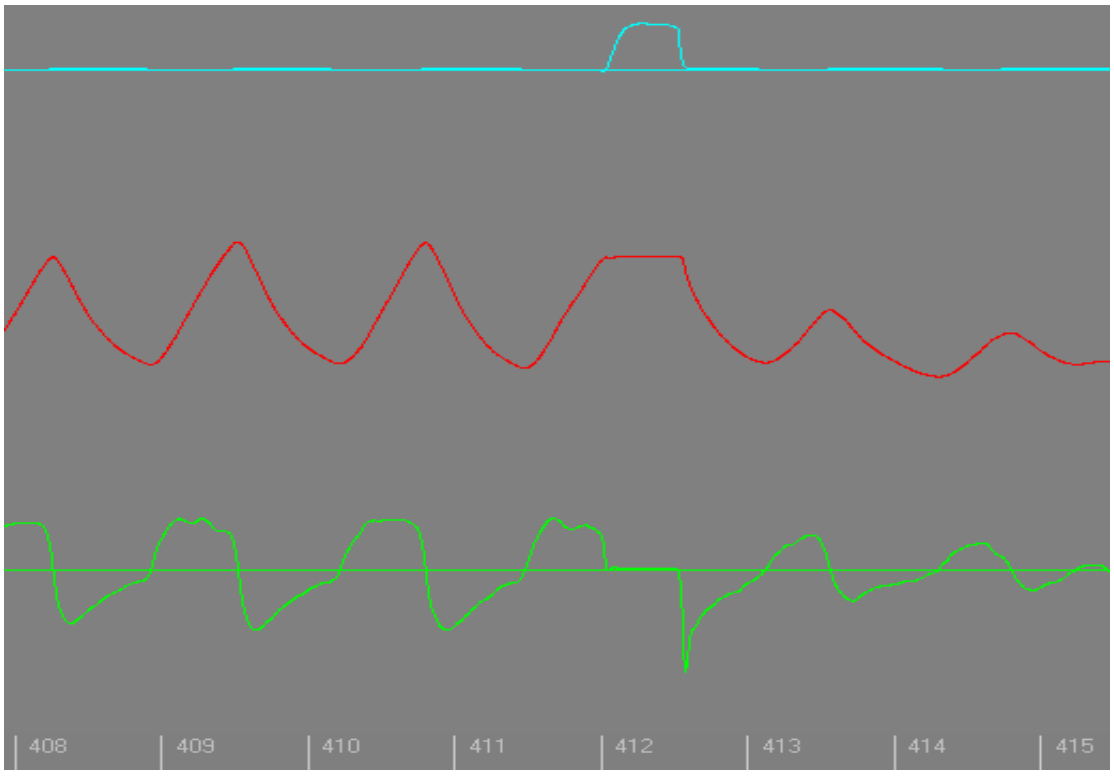
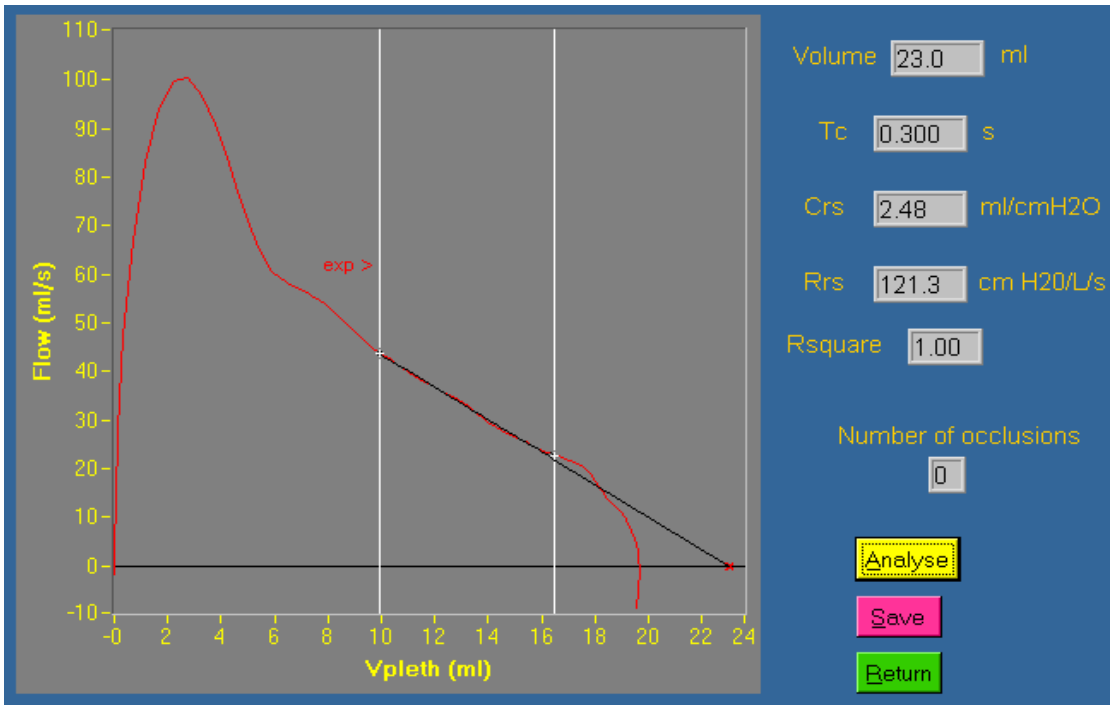


Figure 2.9: The resulting flow/volume loop from Figure 2.8 and the measurement of C_{rs} and R_{rs}



2.4 Lung function at one year corrected age

Infants attended the Amanda Smith lung function laboratory for lung function testing at one year corrected age. If they were clinically well and had been free of respiratory symptoms for at least three weeks they underwent lung function testing. Infants were sedated with 80 mg/kg of Chloral hydrate and were tested supine and naked except for a nappy. The infants were monitored by pulse oximetry (Datex-Ohmeda 3800, Hatfield, UK) throughout the lung function testing and afterwards until they were awake. FRC by helium gas dilution, FRC and LCI by the MBW technique and C_{rs} and R_{rs} by the single breath occlusion technique were measured as at 36 weeks PMA (section 2.3). In addition, whole-body plethysmography was performed.

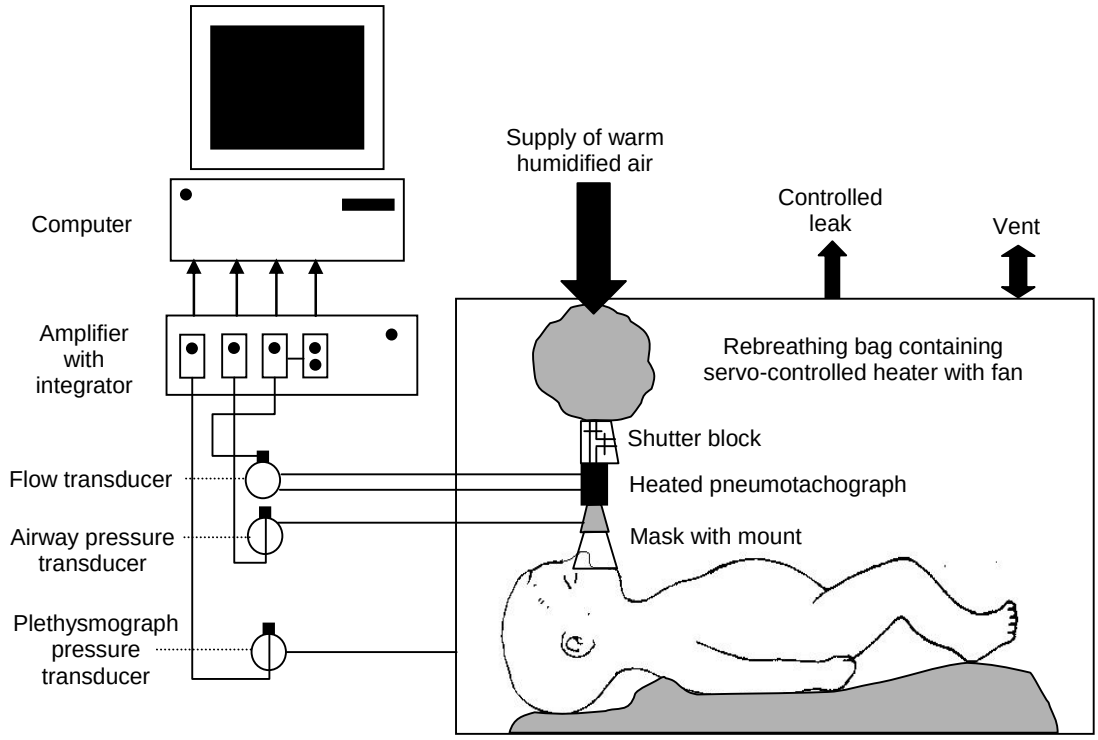
2.4.1 Whole-body plethysmography

2.4.1.1 Equipment

A custom built infant plethysmograph (Department of Medical Engineering, Hammersmith Hospital, London, UK) with a total volume of 90 L, and containing a heated, humidified, re-breathing bag system was used (Figure 2.10).

Figure 2.10: Schematic diagram of the equipment and transducers for the plethysmograph

Adapted from (Thomas et al. 2002).



The constant volume plethysmograph had a fixed leak which improved thermal and pressure stabilisation characteristics. The plethysmograph contained a re-breathing bag system, which consisted of a four litre highly compliant, heat resistant, plastic re-breathing bag. The distal end of the bag was connected to a perspex ring on the top of the plethysmograph which held the bag open and also had a valve, through which the gas in the bag could be refilled with warmed, humidified air between measurements. The proximal end of the bag was connected to a shutter block, which allowed the infant to be switched from breathing air inside the box to the air inside the bag, and a second valve which allowed airway occlusions to be performed. The shutter block was connected to a Fleisch pneumotachograph (Fleisch,

Hechinger, Germany) and mask connector. Suspended within the re-breathing bag was a thermostatically controlled heating element and fan to circulate the air.

Pressure at the airway opening was measured by a differential pressure transducer (range ± 10 kPa, MP 45, Validyne engineering, California, USA) connected to a port in the facemask connector, which also incorporated a thermistor to measure airway temperature. The facemask connector was attached to a heated Fleisch pneumotachograph (Fleisch, Hechinger, Germany) from which airflow was measured. The pneumotachograph was connected to a differential pressure transducer (range ± 0.2 kPa, MP 45, Validyne engineering, California, USA). Pressure changes within the plethysmograph were measured by a differential pressure transducer (range ± 0.2 kPa, MP 45, Validyne engineering, California, USA). All signals were amplified (CD18 carrier amplifiers, Validyne engineering, California, USA) and the flow signal was integrated electronically to give volume (FV 156 Integrator, Validyne engineering, California, USA). The resultant four channels of data were acquired, analysed and displayed in real time on a computer (Gateway, Dublin, Ireland) running a custom written software program (Bodybox, Medical Engineering, Keighley, UK) with analogue-to-digital sampling at 200 Hz (PC-LPM-16PnP, National Instruments, Texas, USA). Equipment (including facemask) dead space volume (41.5 mL, measured by water displacement) was automatically subtracted by the computer software in the calculation of FRC_{pleth}. A vent in the wall of the

plethysmograph was opened between measurements to improve the speed of thermal stabilisation between measurements.

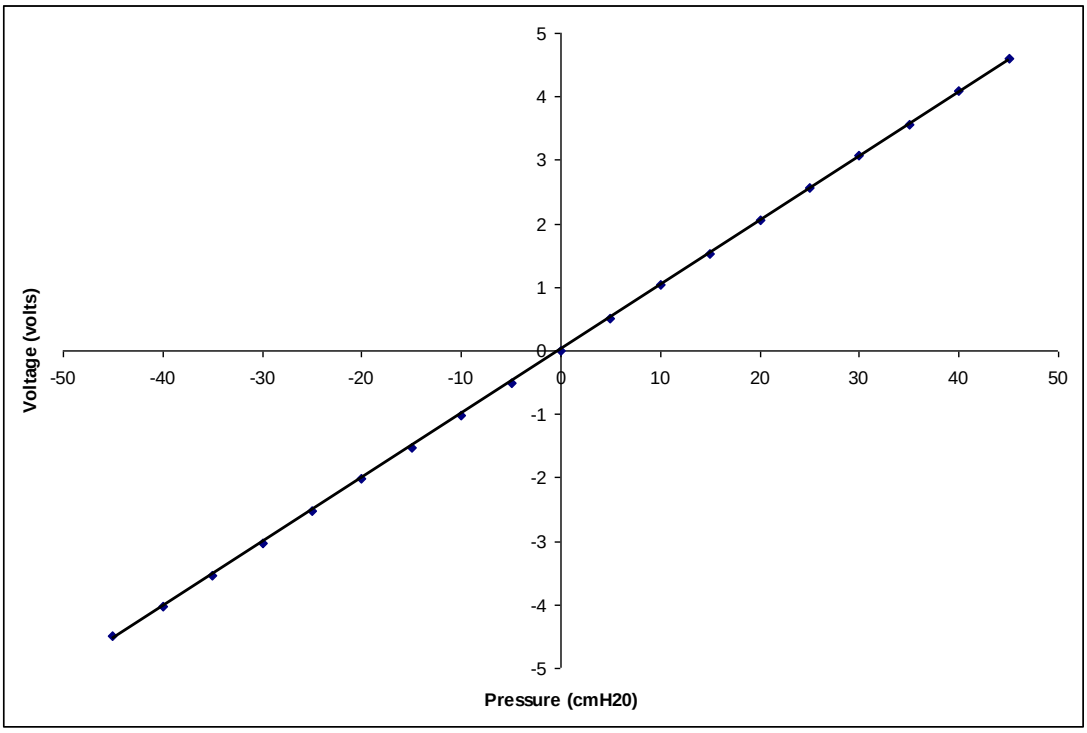
2.4.1.2 Calibration

All channels were calibrated before each measurement. Airway pressure was calibrated using a digital pressure meter (Comark C9505IS, Welwyn Garden City, UK). Flow through the pneumotachograph was calibrated using a flow meter (0-12 L/min, Platon, Roxspur M&C Ltd, Hants, UK) and volume was calibrated with a 100 mL calibration syringe (5510, Hans-Rudolph Inc, Montana, USA). Box pressure was calibrated in terms of volume. Bags of saline equivalent to the patient's weight were placed inside the plethysmograph and an electric, sinusoidal pump (Parvalux electric motors Ltd, Bournemouth, UK) with a stroke volume of 19.2 ml was attached to the plethysmograph and pumped air in and out resulting in a pressure change within the plethysmograph.

2.4.1.3 Validation of transducer linearity

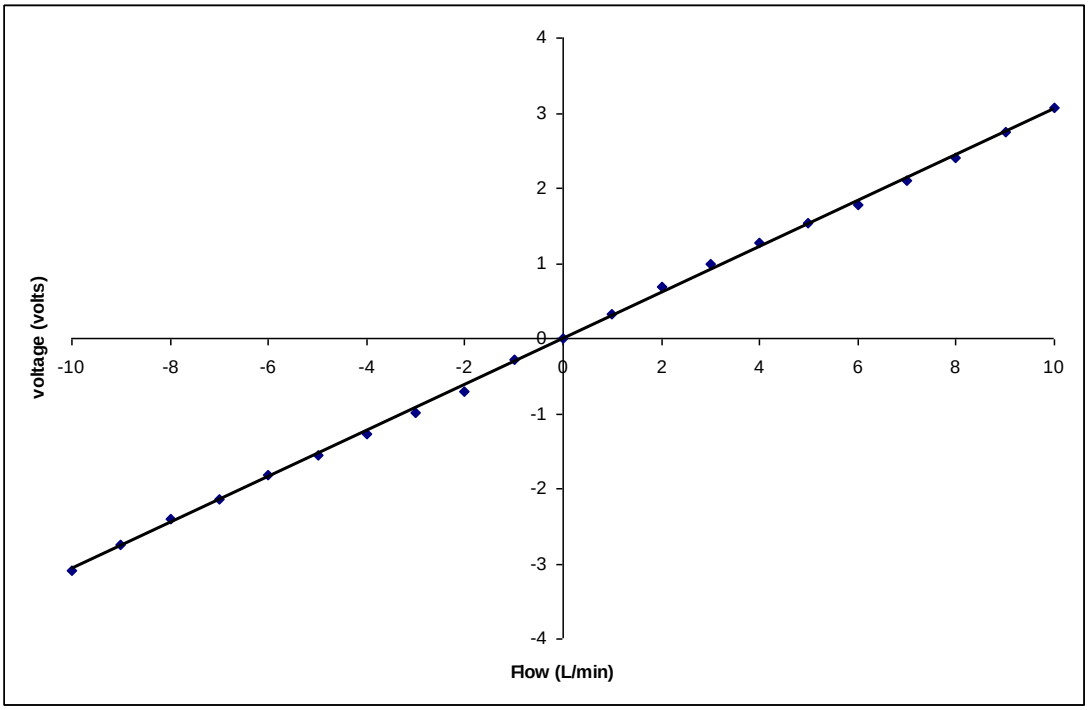
The airway pressure transducer was assessed for linearity over the range of ± 45 cmH_2O using a digital pressure meter (Comark C9505IS, Welwyn Garden City, UK). Applied pressure was compared to the voltage output from the amplifier using BioBench software (version 1.0, National Instruments, Texas, USA). The airway pressure transducer had a linear response (r^2 >0.99) (Figure 2.11).

Figure 2.11: Plot of pressure against voltage produced by the airway pressure transducer



The flow transducer and pneumotachograph were similarly assessed for linearity using a flow meter (0-12 L/min, Platon, Roxspur M&C Ltd, Hants, UK) attached to the pneumotachograph. Inspiratory and expiratory flows were measured over the range 10 L/min in both directions (inspiratory and expiratory). The flow transducer and pneumotachograph had a linear response (r^2 >0.99) (Figure 2.12).

Figure 2.12: Plot of flow against voltage produced by the pneumotachograph and associated flow transducer



2.4.1.4 Leak measurement

The plethysmograph had a small leak to the atmosphere to assist thermal and pressure equilibration. The incidence of slowly occurring pressure changes such as thermal drift due to the re-breathing bag heater and body heat unrelated to respiratory movements could thus be dampened. Rapidly injecting 50 mL of air into the plethysmograph and measuring the time taken for the plethysmograph pressure transducer reading to return to baseline measured the time constant of the leak. The time constant (the time taken for the pressure change to reduce by 63%) was 11.7 seconds. This was within the range recommended by the ERS/ATS guidelines; 10 - 14 seconds (Stocks et al. 2001).

2.4.1.5 Assessment of the frequency response of the system

The frequency response of the pressure transducers and the plethysmograph were measured by balloon burst experiments. It has been recommended that the frequency response for lung function equipment should be at least 10 Hz (Frey et al. 2000). To measure the frequency response of the airway pressure transducer a balloon was connected to the transducer using the tubing employed in the plethysmograph and the pressure changes during the balloon bursts were recorded using Chart software (ADInstruments Pty Ltd, Castle Hill, Australia) with analogue-to-digital sampling at 40kHz (Powerlab 16s, analogue-to-digital converter, ADInstruments Pty Ltd, Castle Hill, Australia). Frequency response = 1/3*Tr*, the response time was five milliseconds, and therefore the airway pressure transducer frequency response was 66.7 Hz.

The frequency response of the box pressure transducer/plethysmograph was measured by bursting a balloon inside the plethysmograph. The balloon was inflated through the port in the top panel of the plethysmograph while leaving the vent open. Once the balloon was inflated, the vent was closed creating a positive pressure within the plethysmograph. The balloon was partially inside and partially outside the plethysmograph, therefore forming a seal around the port it passed through. The balloon was then burst and the pressure change within the plethysmograph as measured by the box pressure transducer was recorded using Chart software (ADInstruments Pty Ltd, Castle Hill, Australia) with analogue-to-digital sampling at 40kHz (Powerlab 16s, analogue-to-digital

converter, ADInstruments Pty Ltd, Castle Hill, Australia). The response time of the pressure transducer/plethysmograph was 23 milliseconds and therefore the total frequency response was 14.5 Hz.

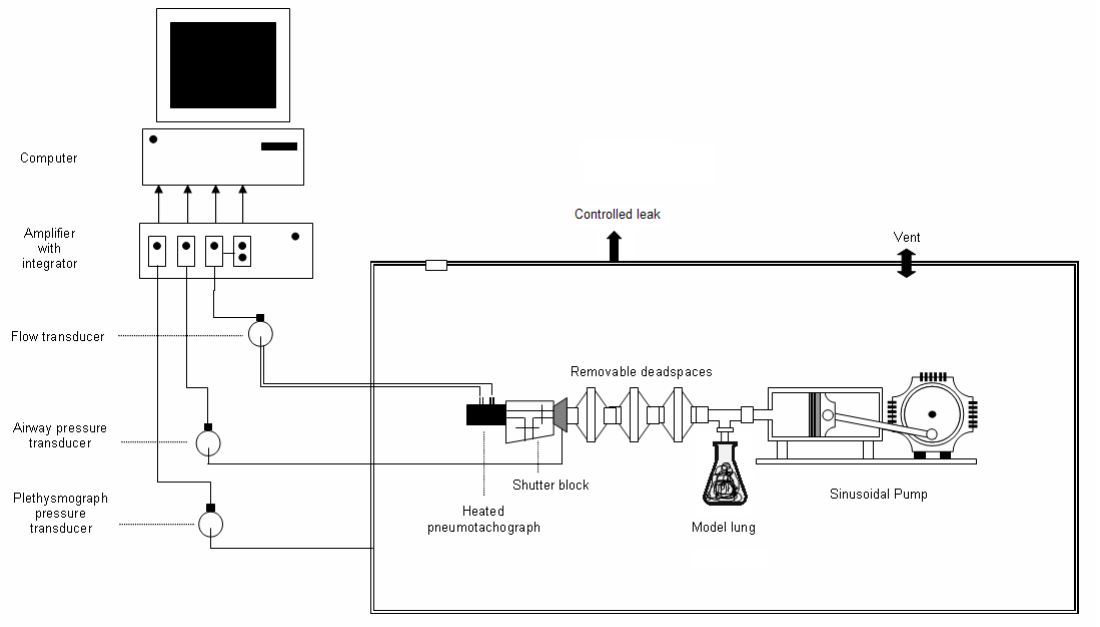
The flow transducer/plethysmograph frequency response was measured as for the box pressure transducer/plethysmograph as above. The balloon was inflated through the port in the top panel of the plethysmograph while leaving the vent open. Once the balloon was inflated, the vent was closed creating a positive pressure within the plethysmograph. The balloon was partially inside and partially outside the plethysmograph, therefore forming a seal around the port it passed through. The balloon was allowed to slightly deflate giving a constant flow, and was then burst and the drop in flow across the pneumotachograph was recorded using Chart software (ADInstruments Pty Ltd, Castle Hill, Australia) with analogue-to-digital sampling at 40kHz (Powerlab 16s, analogue-to-digital converter, ADInstruments Pty Ltd, Castle Hill, Australia). The response time of the flow transducer/plethysmograph was 27 milliseconds and therefore the total frequency response was 12.3 Hz.

2.4.1.6 *In vitro* accuracy of the measurement of volume

The accuracy of the measurement of volume by the plethysmograph was assessed using a lung model which consisted of a glass bottle attached to the pneumotachograph via the mask support using a T-shaped connector (Figure 2.13).

Figure 2.13: Schematic diagram of the lung model within the plethysmograph

Adapted from PhD thesis by S. Broughton, King's College London, 2009.

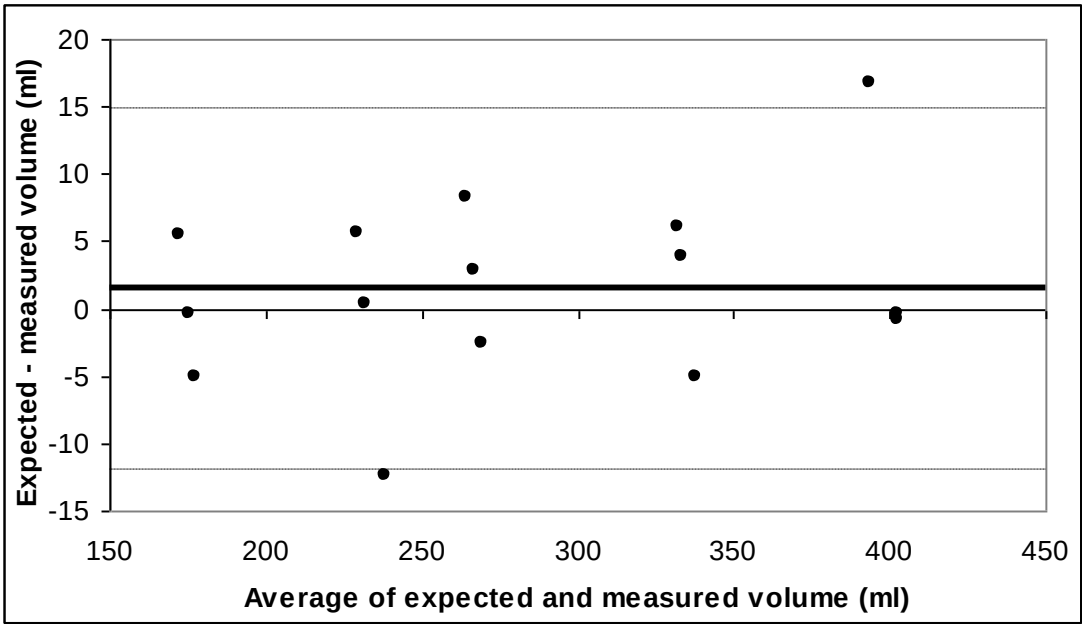


The volume of the lung model was varied by the addition of identical spacers between the T-shaped connector and the mask support. The volume of the lung model was determined by water displacement. Fine copper wire wool was placed inside the lung model to minimise the adiabatic effect. A sinusoidal motorised pump (Parvalux electric motors Ltd, Bournemouth, UK) with a stroke volume of nine mL and a rate of 30 cycles per minute was attached to the lung model. At the start of testing, the plethysmograph was sealed, the electric pump was started and the vent closed. No measurements were made for ten minutes to allow temperature equilibration within the plethysmograph. An occlusion was performed when the piston of the pump was at the maximum inspiratory position and was maintained for three cycles of the pump. The signals were viewed on the computer screen to ensure the changes in box and lung model pressures were in phase and

that at the time of occlusion there was no flow through the pneumotachograph. The measured volume of the lung model was calculated by relating the pressure within the lung model to the pressure changes within the plethysmograph. The plethysmograph was tested at five volumes (175, 232, 268, 335 and 402 ml), selected to encompass the range of lung volumes expected in infants at one year corrected age. The mean (SD) difference in the expected and measured volumes was 1.5 (6.8) mL, which is a smaller volume than would be clinically meaningful, demonstrating good agreement between the measured and expected volumes (Figure 2.14).

Figure 2.14: Bland and Altman plot of expected and measured volumes (mL) for the plethysmograph

The solid line is the mean value and the dotted lines ± 1.96 standard deviations.



2.4.1.7 *In vitro* accuracy of the measurement of resistance

The lung model was identical to that used to assess the accuracy of volume by the plethysmograph (section 2.4.1.6, Figure 2.13) except known calibrated resistances (Hans Rudulph Inc, Kansas City, Missouri, USA) of 20 and 50 $cmH_2O/L/sec$ were placed between the lung model and the mask connector either individually or in series to create resistances of 20, 50 and 70 $cmH_2O/L/sec$. The lung volume of the model used was 288 mL, equivalent to the FRC of an infant at one year corrected age.

For each resistance measured, ten acceptable measurements (see section 2.4.1.8) were recorded. The computer software calculated the resistance from the inspiratory flow trace from 0 to 50% of peak flow (Thomas et al. 2002) and the coefficient of variation was calculated for each set of measurements (Table 2.2).

Table 2.2: *In vitro* results of the resistance of the lung model

Data are presented as mean (SD) or %.

Added resistance	Measured resistance	Co-efficient of variation
20 $cmH_2O/L/s$	20.9 (1.4)	6.7%
50 $cmH_2O/L/s$	51.1 (0.8)	1.6%
70 $cmH_2O/L/s$	71.2 (2.4)	3.3%

2.4.1.8 *In vivo* assessment of FRC_{pleth} and airway resistance

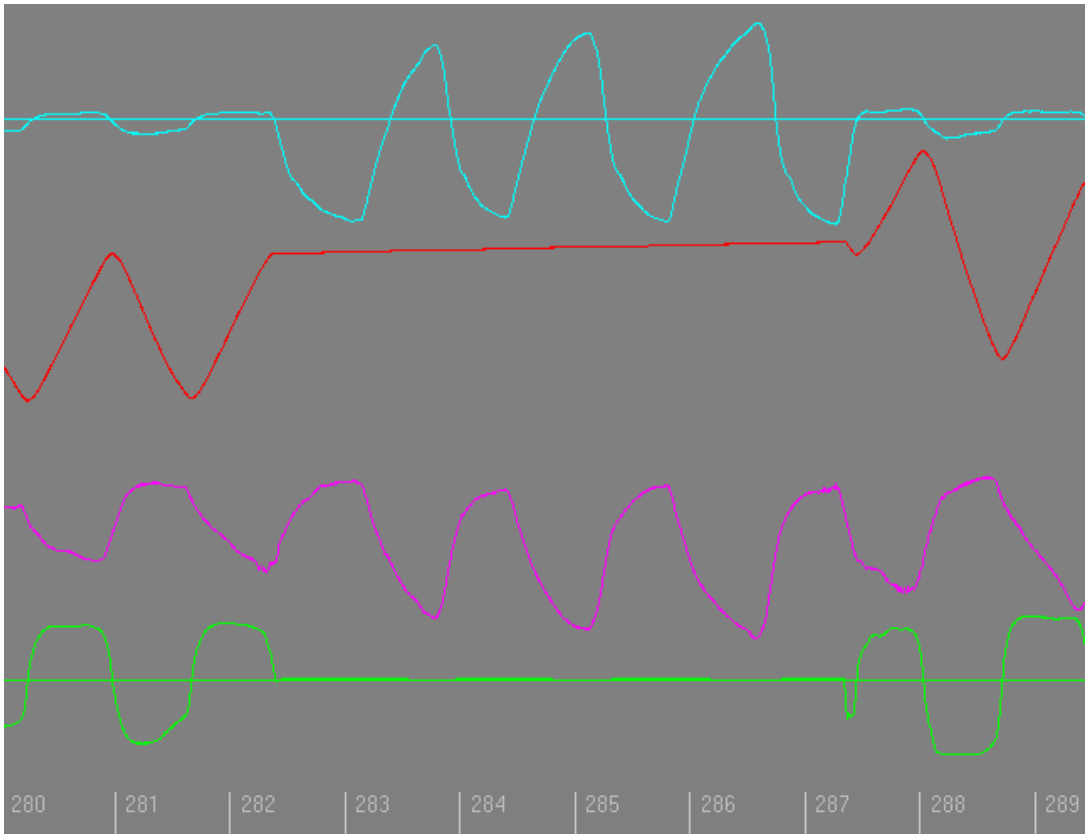
The infant was placed supine inside the plethysmograph and the facemask (Rendell Baker, Laerdal, Norway) with a rim of silicone putty (Promedics, Port Glasgow, UK) was placed on the infant's face covering the mouth and nose (Figure 2.10). The FRC_{pleth} was calculated from a minimum of three end-inspiratory occlusions. End-inspiratory occlusions, rather than end-expiratory, were used as they have been shown to result in less glottic activity during respiratory efforts against the occlusion (Yüksel & Greenough 1994). At the time of the occlusion FRC_{pleth} is calculated from the equation:

FRC_{pleth} = (box pressure/airway pressure) x atmospheric pressure

Occlusions were considered acceptable if the V_{pleth} and airway pressure were in phase and no airflow was evident during the occlusion (Figure 2.15).

Figure 2.15: Data from the plethysmograph showing an end inspiratory occlusion

The blue line is airway pressure, the red line is airway volume, the pink line is plethysmograph volume and the green line is airway flow.

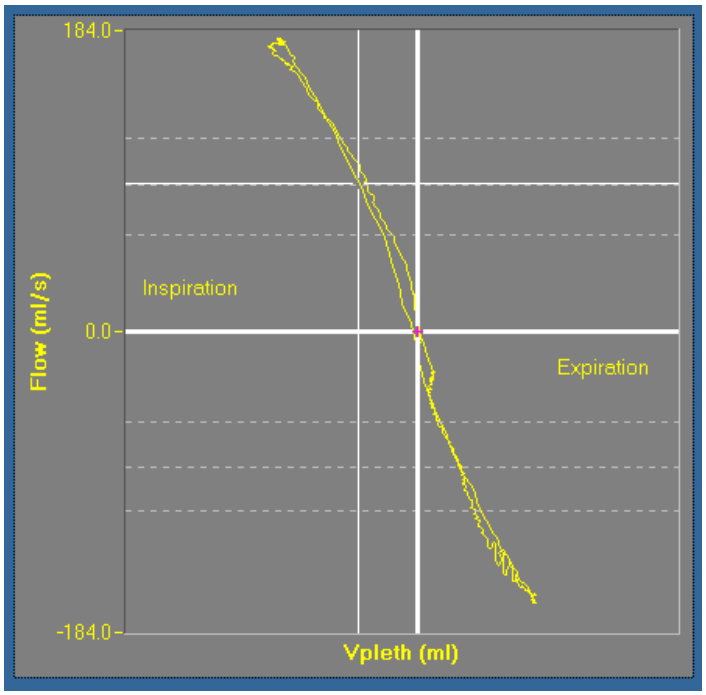


Seventy two infants had acceptable FRC_{pleth} measurements, the mean intra-subject co-efficient of variation for FRC_{pleth} was 3.2 %.

After measuring FRC_{pleth}, airway resistance (R_{aw}) was measured. The infant's tidal breathing was diverted to the heated and humidified re-breathing bag. Individual breaths acquired during periods of re-breathing from a single recording were displayed as x/y plots of V_{pleth}/flow by the computer. Only technically acceptable breaths, that is the loop was closed or nearly closed at points of zero flow, were used in the analysis (Stocks et al. 2001). Inspiratory

R_{aw} was calculated electronically by applying a regression line to the portion of the loop between 0 and 50% maximal inspiratory flow (Thomas et al. 2002) (Figure 2.16).

Figure 2.16: An x/y plot of Vpleth/flow during re-breathing, showing measurement from 0-50% of maximum inspiratory flow



During all R_{aw} measurements, the computer calculated the apparatus resistance of the selected portion of the individual breath by relating the change in airway pressure to the change in flow and then subtracted this value from the total measured resistances (Thomas et al. 2002). At least fifteen resistance results were obtained from each infant.

The reproducibility of FRC_{pleth} and R_{aw} was tested on five infants with two sets of tests carried out on the same day. These results demonstrated good

short term reproducibility with a co-efficient of variation percentage (standard deviation) of 3.6% (4.1) and 14.6% (5.3) respectively and a mean difference of 1.5 mL/Kg and 0.8 $cmH_2O/L/s$ respectively.

2.5 Healthcare utilisation and cost of care analysis

2.5.1 Neonatal healthcare utilisation and cost of care

Infants' and their mothers' hospital notes and the King's College Hospital (KCH) neonatal database were reviewed to find the number of days each infant spent at each level of neonatal care (i.e. intensive care [ICU], high dependency [HDU], special care [SCBU] or maternity ward) as defined by the British Association of Perinatal Medicine guidelines (British Association of Perinatal Medicine 2001). Maternity ward costs included days the mother and infant stayed on a postnatal ward for a reason relating to the infant (e.g. intravenous antibiotics, phototherapy, blood sugar monitoring) but not those relating to the mother (e.g. blood pressure monitoring, wound care post caesarean section). Costs (UK £) for each level of care were obtained from the NHS reference costing scheme (2007-8) (£974, £750, £421, and £474 for ICU, HDU, SCBU and maternity ward respectively) (Department of Health 2009), thus:

Total neonatal costs = (days ICU x £974) + (days HDU x £750) + (days SCBU x £421) + (days maternity ward x £474).

2.5.2 Follow up healthcare utilisation and cost of care

At one (both cohorts) and two (2008 cohort only) years corrected age, the infants' hospital and GP records were examined to determine healthcare utilisation during infancy. Hospital records were examined for hospital re-admissions, Accident and Emergency (A and E) attendances, out-patient attendances and any medications prescribed. GP records were examined for hospital re-admissions, A and E attendances, number of out-patient hospital appointments, number of GP attendances, number of referrals to community support services and all medication prescribed. All visits to practice nurses or routine visits to health visitors, for example for immunisations, were not recorded as these were considered the usual costs for infants. Follow up costs after neonatal discharge were calculated using the NHS reference costing scheme (2007-8) (Department of Health 2009) and the British National Formulary for Children (BNFC) (Royal College of Paediatrics and Child Health 2008). The NHS reference costing scheme gives national average costs for in- and out-patient hospital attendances and GP attendances (Department of Health 2009). This includes all costs routinely associated with the diagnosis requiring attendance (e.g. medical and non-medical personnel, medication used during the admission, medication given to the patient on discharge and medical equipment). For in-patient admissions the number of days for each admission was multiplied by the national average cost for the diagnosis leading to admission. A respiratory attendance to hospital or GP was defined as an appointment with a healthcare professional relating to a LRTI (i.e. not an URTI). From the BNFC

the lowest price relating to the medicine prescribed was used. Daily home oxygen costs were obtained from the local authority the patient lived in. Palivizumab was considered an out-patient rather than a neonatal cost.

Potential sources of bias when examining health care costs include the incorrect classification of diagnoses leading to incorrect attributable costs, the use of average costs from the NHS reference costing scheme may not be appropriate for the local healthcare service, the difficulty in the statistical analysis of heavily skewed data and time-horizon bias, which should be avoided as infants will be followed up to one or two years corrected age.

2.6 Respiratory health related questionnaires and diary cards

Parents completed a diary card (Appendix 1) for one month when their infant was 11 (both cohorts) and 23 (2008 cohort only) months corrected age, indicating each day whether their infant coughed, wheezed, used any respiratory related medications (e.g. inhalers, oral steroids, antibiotics) or visited any healthcare professional for an acute problem (i.e. not including routine out-patient appointments). In addition, parents completed a respiratory health related questionnaire (Appendix 2) about their infant at one (both cohorts) and two (2008 cohort only) years corrected age.

The internal validity of the respiratory-related questionnaire (for the questions “has you child coughed in the last year”, “has your child wheezed in the last year”, “has you child used any respiratory medications”, “has your child used

bronchodilators, preventers or antibiotics" and "has your child been diagnosed with asthma") was assessed by calculating Cronbach's alpha using IBM SPSS Statistics (version 19, New York, USA). The Cronbach's alpha was 0.753 which indicated a good level of internal consistency for the respiratory related questionnaire. Removal of either of the questions "has you child coughed in the last year" or "has your child been diagnosed with asthma" would have minimally improved the internal validity of the questionnaire (increasing the Cronbach's alpha to 0.756 or 0.769 respectively).

The internal validity of the diary card (using all five questions) was assessed by calculating Cronbach's alpha using IBM SPSS Statistics (version 19, New York, USA). The Cronbach's alpha was 0.625 which indicated an acceptable level of internal consistency for the diary card. Removal of any of the questions would not have improved the overall validity of the diary card.

2.7 Virus identification

Nasopharyngeal aspirates (NPAs) were obtained from infants with signs of a LRTI, either in the community or in hospital. Approximately one mL of normal saline was gently instilled into each nostril of the infant. After approximately one minute a size eight or ten suction catheter (Unomedical, Worcs, UK), connected to a tracheal suction set (Unomedical, Worcs, UK), was inserted approximately five cm into each nostril in turn and the suction unit (Laerdel, Stavanger, Norway) turned on. The saline and any mucus was suctioned

from each nostril as the catheter was slowly withdrawn. Up to five mL of 0.9% saline was then suctioned through the catheter to move any mucus stuck in the catheter into the tracheal suction set. The sample was then stored at 4^{o}C until processed.

2.7.1 Nucleic acid extraction

The NPA was mixed with sterile Phosphate buffered saline (PBS), and any mucus broken up with a Pasteur pipette. This was then centrifuged to 3000 x *g* for ten minutes. The mucus and excess PBS was poured off and the pellet produced was resuspended in one mL of lysis buffer (QIAGEN, Crawley, UK), dispensed into a Sarstedt vial, and stored at 4^{o}C until processed.

Nucleic acid was extracted from the samples using the commercially available QIAsymphony cabinet and QIAsymphony virus midi kit (QIAGEN, Crawley, UK). It was possible to extract nucleic acid from 96 samples per run. A negative control (RNA free water) was included in every 24 samples to test for cross contamination. The sample (400 μL) initially underwent lysis to inactivate the RNases and obtain intact viral nucleic acid. The sample was then buffered and the nucleic acid bound to magnetic particles. These were then washed off using another buffer and finally the nucleic acid (120 μL) was eluted in RNase free buffer. This was stored at -80^{o}C until PCR was undertaken.

2.7.2 Real-time reverse transcriptase PCR

2.7.2.1 Previously developed real-time PCR assays

Three multiplex assays and two monoplex assays were already available and in routine clinical use at the commencement of this thesis (Heim et al. 2003; Auburn et al. 2011). All NPAs were initially tested for ten viruses by the KCH virology department as routine clinical work. Nine RNA viruses were tested by real time reverse transcriptase PCR (rt RT-PCR) in three multiplexes with a monoplex RNA internal control (IC) as described by Auburn et al (2011). The multiplexes tested were:

Multiplex 1: Influenza A and B and human metapneumovirus

Multiplex 2: Parainfluenza virus types 1, 2 and 3

Multiplex 3: RSV A, RSV B and rhinovirus

Monoplex: RNA internal control

The primers were all used at five pmoles and the probes at one pmole. The samples were tested using a Rotor-Gene 6000 real-time thermal cycler (QIAGEN, Crawley, UK) and the following conditions:

1. Hold at 50^{o}C for 30 minutes (reverse transcription)
2. Hold at 95^{o}C for 15 minutes (TaqMan polymerase activation and reverse transcriptase inactivation)
3. 50 cycles of:
    a. Step 1- 95^{o}C hold for 30 secs (denaturation)

b. Step 2- 57^{o}C hold for 30 secs, acquiring to cycle A (FAM, IRD700, HEX, ROX) (annealing and extension)

Adenovirus (DNA virus) was tested by real-time PCR (rt-PCR) in monoplex along with a DNA IC. The adenovirus primers were used at ten pmoles and the probe at two pmoles. The DNA IC primers were used at five pmoles and the probe at one pmole. The samples were tested using a Rotor-Gene 6000 real-time thermal cycler (QIAGEN, Crawley, UK) and the following conditions:

1. Hold at 95^{o}C for 15 minutes
2. 45 cycles of:
    a. Step 1- 95^{o}C hold for 15 secs
    b. Step 2- 57^{o}C hold for 20 secs, acquiring to cycle A (Cy5, ROX)
    c. Step 3- 65^{o}C hold for 20 secs

Each reaction included the IC, allowing monitoring of each sample for potential inhibition. This prevented the reporting of false negative results, since in a true negative the internal control will amplify, whereas if the internal control fails to amplify, this would potentially indicate inhibitory compounds in the sample, also preventing the amplification of the nucleic acid from any potential pathogen in the sample.

2.7.2.2 Development of a new multiplex real-time PCR assay

A new multiplex rt-PCR assay was developed to identify the DNA virus human bocavirus (HBOV), the two RNA viruses enterovirus (EV) and

parechovirus (PEV), and a bacteriophage MS2 RNA IC. The primers and probes (Metabion, Martinsried, Germany) used in the assays were previously published and are shown in Table 2.3.

The primers and probes were initially tested in monoplex to ensure they identified the target sequence and the product size was confirmed by post PCR agarose gel electrophoresis (Figure 2.17).

Figure 2.17: Agarose gel plate demonstrating detection of a PEV DNA product

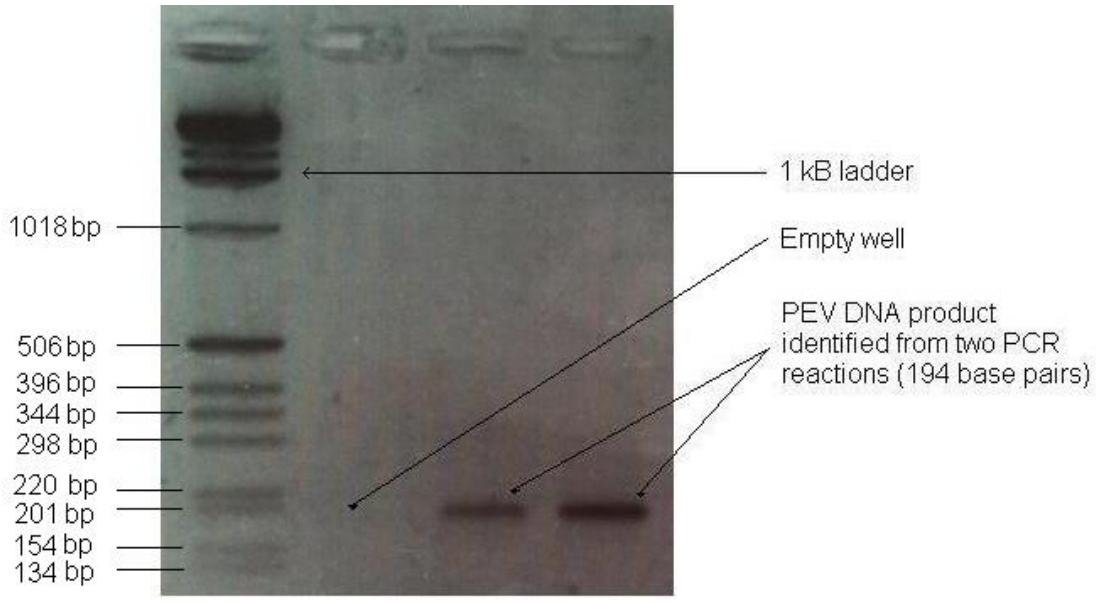


Table 2.3: Sequences of the primers and probes used for the new multiplex real-time (reverse transcriptase) PCR assay

Virus	Primer/Probe	Sequences and labels	Reference
HBOV (NP1)	Forward primer	5'-AGA GGC TCG GGC TCA TAT CA-3'	(Lu et al. 2006)
	Reverse primer	5'-CAC TTG GTC TGA GGT CTT CGA A-3'	(Lu et al. 2006)
	TaqMan Probe	HEX-AGG AAC ACC CAA TCA RCC ACC TAT CGT CT-BHQ3	(Lu et al. 2006)
EV	Forward primer	5'-CAT GGT GYG AAG AGT CTA TTG AGC TA-3'	(Brittain-Long et al. 2008)
	Reverse primer	5'-GGA CAC CCA AAG TAG TCG GTT C-3'	(Brittain-Long et al. 2008)
	TaqMan Probe	FAM-CGG CCC CTG AAT GCG GCT AAT C-BHQ1	(Brittain-Long et al. 2008)
PEV	Forward primer	5'-GTA ACA SWW GCC TCT GGG SCC AAA AG-3'	(Nix et al. 2008)
	Reverse primer	5'-GGC CCC WGR TCA GAT CCA YAG T-3'	(Nix et al. 2008)
	TaqMan Probe	IRD700-CCT RYG GGT ACC TYC WGG GCA TCC TTC-BHQ3	(Nix et al. 2008)
MS2 IC	Forward primer	5'-TGG CAC TAC CCC TCT CCG TAT TCA CG-3'	(Rolfe et al. 2007)
	Reverse primer	5'-GTA CGG GCG ACC CCA CGA TGA C-3'	(Rolfe et al. 2007)
	TaqMan Probe	ROX-CAC ATC GAT AGA TCA AGG TGC CTA CAA GC-BHQ2	(Rolfe et al. 2007)

The sequences for the primers and probes for HBOV were obtained from the study by Lu et al (2006). That study (Lu et al. 2006) developed two real-time PCR assays targeting two HBOV genes; NP1 and NS1. Prior to deciding which assay to use in the multiplex we tested 23 known positive HBOV samples from the virology laboratory at the Royal Infirmary Hospital in Edinburgh (RIE) and compared the cycle threshold (Ct) values from the two assays. Those results were then compared with the Ct results from RIE's different assay that targeted the NS1 gene. The results of these comparisons are shown in Table 2.4.

Table 2.4: Comparison of the Ct values of the assays for HBOV

	(Lu et al. 2006)		RIE
Sample Number	NP1	NS1	NS1
1	35.09	34.70	38.57
2	34.81	32.84	36.27
3	28.08	37.29	39.87
4	30.93	30.23	34.15
5	39.17	36.60	42.71
6	33.90	31.78	36.66
7	26.72	24.93	28.50
8	31.50	29.56	32.74
9	19.55	18.44	21.04
10	33.13	32.11	35.63
11	38.19	Not detected	42.33
12	36.21	37.46	39.52
13	38.61	36.77	38.96
14	27.80	27.72	28.05
15	35.12	33.31	35.95
16	38.65	34.92	42.78
17	38.53	35.27	41.60
18	37.76	34.59	37.30
19	36.10	43.21	38.98
20	36.40	36.89	41.90
21	38.50	Not detected	41.58
22	36.23	Not detected	38.84
23	36.43	34.06	39.10

As the assay targeting the NP1 gene identified every sample and had Ct values that were lower for every sample than the Edinburgh assay we decided to use the NP1 assay in the multiplex (Table 2.3). The primers and probes for HBOV (NP1), EV, PEV, and MS2 were combined into a multiplex which was demonstrated to detect all four targets without any inhibition.

2.7.2.3 PCR protocol and methodology

For HBOV, EV, and PEV the primers were all used at five pmoles and the probes at three pmoles. For the MS2 bacteriophage the primers and probe were all used at two pmoles. Real-time PCR was performed using QIAGEN Quantitech Multiplex RT-PCR kits (QIAGEN, Crawley, UK). The multiplex reaction mix consisted of 4 μL of detection mix (1 μL [5 pmoles of each primer and 3 pmoles of each probe] of each of the HBOV, EV, and PEV monoplex detection mixes, and 1 μL [2 pmoles of each primer and probe] of the MS2 bacteriophage monoplex detection mix), 12.5 μL of RT-PCR reaction mix, 0.5 μL of QIAGEN RT enzyme, 1 μL of MS2 bacteriophage RNA and 7 μL of purified nucleic acid template, giving a total reaction volume of 25 μL.

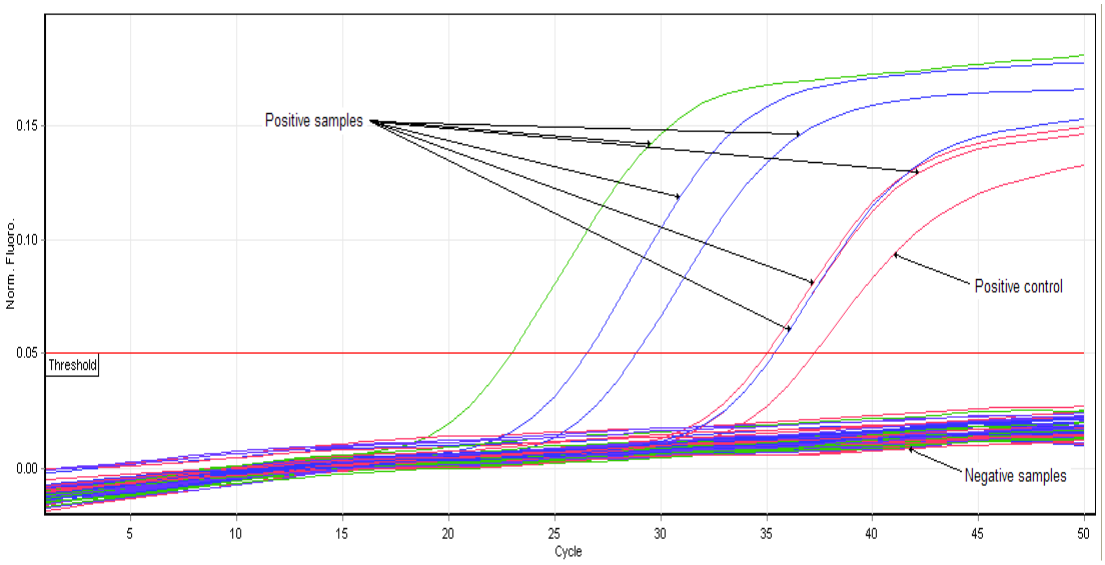
The samples were tested using a Rotor-Gene 6000 real-time thermal cycler (QIAGEN, Crawley, UK) and the following conditions:

1. Hold at 50^{o}C for 30 minutes (reverse transcription)
2. Hold at 95^{o}C for 15 minutes (TaqMan polymerase activation and reverse transcriptase inactivation)

3. 50 cycles of:
    a. Step 1- 95^{o}C hold for 15 secs (denaturation)
    b. Step 2- 60^{o}C hold for 45 secs, acquiring to cycle A (FAM, IRD700, HEX, ROX) (annealing and extension)

All the negative controls (RNA free water) tested negative after rt-PCR suggesting no cross contamination between the samples. All positive samples (Figure 2.18) were re-tested to confirm the initial result and all were still positive on repeat testing. None of the samples demonstrated any inhibition.

Figure 2.18: Example of the output from the Rotor-Gene 6000 for 60 samples tested for HBOV



2.8 Genetic analysis

Blood or buccal swabs were obtained from infants prior to neonatal discharge. Either one mL of blood was collected into an EDTA tube and stored at -20^{o}C until testing, or three buccal swabs (Heinz-Herenz, Hamburg, Germany) were obtained at least 30 minutes after feeding by firmly scraping the inside of each cheek and between the gums and lips. The swabs were placed in a sterile polypropylene tube (Greiner Bio-One GmBH, Gloucs, UK) and stored at -20^{o}C. The samples were then sent on dry ice to the National Institute for Public Health and the Environment (RIVM) in Bilthoven, The Netherlands for testing.

2.8.1 DNA extraction

DNA was isolated from blood samples or buccal swabs. Genomic DNA was extracted from blood by digestion with proteinase K, followed by salting out with potassium acetate and chloroform/isoamyl alcohol extraction. DNA from the buccal swabs was extracted using the QIAcube instrument and the QIAamp DNA Blood Mini Kit (QIAGEN, Venlo, The Netherlands). Nine hundred microlitres of sample gave 120 μL of purified DNA. The DNA concentration was determined using a nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). DNA samples were then diluted with TE Buffer (10 mM Tris, 1 mM EDTA) to give a final concentration of 20 ng/μL (range 10-25 ng/μL). The purified DNA was stored at -20^{o}C until analysed.

2.8.2 Single nucleotide polymorphism analysis

2.8.2.1 Single nucleotide polymorphisms to be analysed

Eleven single nucleotide polymorphisms (SNPs) were chosen to be tested. This number was chosen to ensure sufficient statistical power and to reduce the probability of false positive associations due multiple statistical testing. The SNPs were chosen as they had previously been associated with severe RSV infection, with RSV in premature babies or with prematurity and BPD (Table 2.5). The choice of SNPs to analyse was purposefully delayed until near the end of the study in order to be able to test for any new SNPs that may be found to be relevant to RSV infection during the course of the study.

Table 2.5: SNPs tested and the associated gene and process

SNP	Gene	Process	Association	Reference
rs2787094	A disintegrin and metalloprotease 33 (ADAM33)	Airway remodelling	RSV hospitalisation in premature infants and preschool lung function and wheeze	(Siezen et al. 2009; Simpson et al. 2005)
rs1800872	IL10	Anti-inflammatory cytokine that inhibits T_H1 responses and influences antigen presentation and mast cell proliferation (adaptive immunity)	RSV hospitalisation, RSV hospitalisation requiring ICU and HRV hospitalisation	(Janssen et al. 2007; Hoebee et al. 2004; Wilson et al. 2005; Helminen et al. 2008)
rs2243191	IL19	Member of the IL10 cytokine family (adaptive immunity)	Recurrent wheeze at one year of age after RSV bronchiolitis	(Ermers et al. 2011)
rs334353	Transforming growth factor-β receptor-1 (TGFβR1)	Airway remodelling and adaptive immunity	RSV hospitalisation in premature infants	(Siezen et al. 2009)
rs10735810	Vitamin D receptor (VDR)	Innate immunity	RSV hospitalisation	(Janssen et al. 2007)
rs1060826	Nitric oxide synthase type 2A (NOS2A)	Innate immunity and airways mucosal response	RSV hospitalisation	(Janssen et al. 2007)
rs2233409	Nuclear factor-κ-B 1A (NFκB1A)	Innate immunity	RSV hospitalisation in premature infants	(Siezen et al. 2009)
rs1805723	Killer cell lectin-like receptor subfamily G member 1 (KLRG1)	Regulation of specific humoral and cell-mediated immunity	Prematurity	Unpublished data from Bont et al.
rs2664349	Matrix metalloproteinase-16 (MMP16)	Alveolarisation of the lung	Prematurity and BPD	(Hadchouel et al. 2008)
rs2664352	MMP16	Alveolarisation of the lung	Prematurity and BPD	(Hadchouel et al. 2008)
rs1124	Pulmonary surfactant protein C (SFTPC)	Stabilisation of the phospholipid film	Prematurity and RDS	(Lahti et al. 2004)

2.8.2.2 SNP analysis methodology

Extracted DNA samples were diluted with TE Buffer to 7 ng/µL and sent to KBioscience (Herts, UK) for genotyping with the KASPar technology to test nine SNPs (ADAM33 rs2787094, IL10 rs1800872, IL19 rs2243191, KLRG1 rs1805723, MMP16 rs2664349, MMP16 rs2664352, NFκB1A rs2233409, SFTPC rs1124, and TGFβR1 rs334353). The sequences of DNA containing the SNPs are shown in Table 2.6. Two other SNPs (VDR rs10735810 and NOS2A rs1060826) were tested at the RIVM using the primers and probes shown in Table 2.7 for VDR or a pre-designed assay for NOS2A.

Table 2.6: DNA sequences for the nine SNPs analysed by KBioscience

The base pairs in square brackets are the SNPs analysed.

SNP	Sequence
ADAM33 rs2787094	AGGAAGGAAGGTCCCCAAAATTATGTTTGYTTGCAGAGGCCAGCCAGGCT[C/G]CAGGGGAGTGTGGAYTCAG TCGAACCATAGGGCCCCAGGACCACTAGCTT
TGFβR1 rs334353	ACCCATAATTTTGGTAGGTTTTTGAAGCTTCAGTMTGAGGACTGGCATTC[G/T]TTTGTATCTATTATTTTTTTCC CTGGAGTATATYCGTGCCCTTCCTTTCA
IL10 rs1800872	CTAATGAAATCGGGGTAAAGGAGCCTGGAACACATCCTGTGACCCCGCCTGT[A/C]CTGTAGGAAGCCAGTCT CTGGAAAGTAAAATGGAAGGGCTGCTTGGGAAC
NFκB1A rs2233409	GCCGACGACCCCAATTCAAATCGATCGTGGGAAACCCCAGGGAAAGAAGG[C/T]TCACTTGCAGAGGGACAGG ATTACAGGGTGCAGGCTGCAGGGAAGTACCG
MMP16 rs2664352	CTCTCCACTGTGGGGTAAACCTNGGTAGCGAGGCTCATCAGGCAGTCTCA[C/T]AGGTGAGTGGAGTTTGGAA GGCAGCAGTGAACAGGTAGCTATGTACATCT
MMP16 rs2664349	CAGTCTGCAGATAGGANTACAACCCCAACTTGCCACTAATAAGGGGAGAA[A/G]TAAAAGTTCTGTCTCGAGAT AAGCATAGTCAATTTGCAAAAATTCAGAAG
IL19 rs2243191	AGAGCTCGACGTCTTTCTAGCCTGGATTAATAAGAATCATGAAGTAATGT[C/T]CTCAGCTTGATGACAAGGAAC CTGTATAGTGATCCAGGGATGAACACCCC
SFTPC rs1124	GGCGAGATGCAGGCTCAGCACCCTCCGGAGGGGACCCGGCCTTCCTGGGCATGGCCGTGA[A/G]CACCCTGT GTGGCGAGGTGCCGCTCTACTACATCTAGGACGCCTCCGGTGAGCAGGTGTG
KLRG1 rs1805723	CTTATTCTACTCCCCATTCATCCATAGCCTACTGTTGCTATGCATCGTCTTCCATGCTACCTTGGTTAACTCACA TTTTTGCTGTGGTCC[A/G]TAGTCATTCTGGGCTTGGGTTGCNGTAGGCAACTCTAACATGGAATAAATAACACT GTCAGTCATCTTCAGCTAAGATCTTTCACATGCA

Table 2.7: Primers, probes and assay number used to test for the VDR and NOS2A SNPs

SNP	Assay number	Primers	Probes
VDR rs10735810	N/A	Forward 5'-GGG TCA GGC AGG GAA GTG-3' Reverse 5'-TGG CCT GCT TGC TGT TCT T-3'	VIC-ATTGCCTCCATCCCTGT FAM-TGCCTCCGTCCCTGT
NOS2A rs1060826	C_9458082_10	N/A	N/A

Real-time PCR was performed using TaqMan Genotyping Master Mix (Applied Biosystems, Carlsbad, USA). The reaction mix consisted of 2.5 μL of TaqMan genotyping master mix, 0.25 μL of TaqMan primers and probes (giving a final concentration of 0.9 μM for each primer and 0.2 μM for each probe), 1.25 μL of nuclease-free water, and 1 μL of sample DNA (at a concentration of 20 ng/μL), giving a total reaction volume of 5 μL.

The samples were tested using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, USA) and the following conditions:

1. Hold at 95^{o}C for 10 minutes
2. 40 cycles of:
    a. Step 1- 95^{o}C hold for 15 secs
    b. Step 2- 60^{o}C hold for 60 secs, acquiring channels VIC, FAM and ROX

The genotypes were determined by reading the fluorescent signal of FAM and VIC from the end-products. Figures 2.19 and 2.20 show the scatter plots of the genotyping.

Figure 2.19: Scatter plot of fluorescence signal after rt-PCR for the SNP TGFβR1 rs334353 (from KBioscience)

The three genotypes are visible as three distinct clusters. In the left lower corner all negative controls are visible.

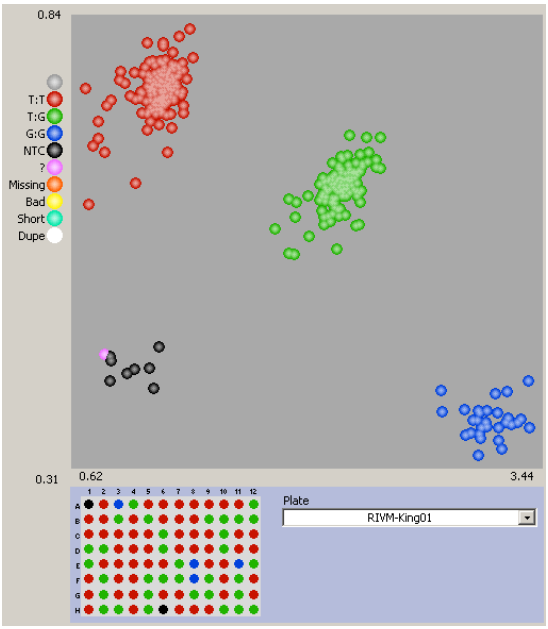
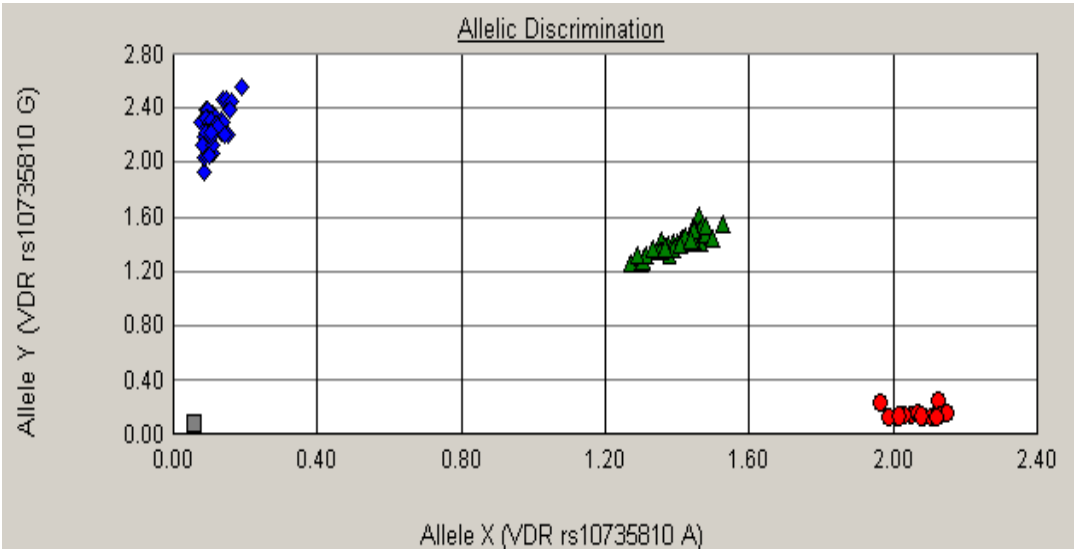


Figure 2.20: Scatter plot of fluorescence signal after rt-PCR for the SNP VDR rs10735810 (from RIVM)

The three genotypes can be seen as separate clusters with the negative controls in the left lower corner.



2.9 Statistical analysis

Continuous data were tested for normality using the Shapiro-Wilk test. Normally distributed data were assessed for statistical significance using the one way ANOVA test and non-normally distributed data using the Kruskal-Wallis test. Multiple comparisons were performed using the one way ANOVA test with a post hoc Bonferroni test or the Kruskal-Wallis test with a post hoc Dunn's or ANOVA test. Categorical data were analysed using either the Chi squared test or Fisher's exact test as appropriate. Statistical analysis was performed using IBM SPSS Statistics (version 19, New York, USA).

Cost of care data were summarised using means, rather than medians to preserve the total sum of costs (Thompson & Barber 2000) and regression models were fitted using generalised linear models (Barber & Thompson 2004) using Stata (version 11.1, Texas, USA).

2.9.1 Sample size calculation

In a previous study (Broughton et al. 2007), 59 parents of 126 infants (46%) who had been recruited into a follow up study (Broughton et al. 2005) consented for their infants to have lung function studies at one year of age. The planned sample size for an overarching study investigating respiratory morbidity following respiratory virus LRTIs was 160 infants, as that would likely yield at least 72 infants whose parents would consent for

them to have lung function assessment at one year. In the previous study (Broughton et al. 2007), there was a difference at one year, between infants who had had or had not had a RSV LRTI equivalent to one standard deviation in the airway resistance results. In that study (Broughton et al. 2007), a greater proportion of parents whose infants who had a RSV LRTI compared to those who had not, consented for their infant to have lung function studies. Assuming a ratio of 2:1, with 72 infants recruited, it would be possible to detect a difference equivalent to 0.7 standard deviations in the airway resistance results with 80% power and at the 5% significance level, that is, a smaller difference than that observed previously (Broughton et al. 2007).

Chapter 3: Genetic predisposition of RSV infection related respiratory morbidity in preterm infants

3.1 Introduction

Respiratory syncytial virus (RSV) infects almost all children by two years of age. Studies have demonstrated that some infants born at term may be genetically predisposed to developing severe RSV lower respiratory tract infections (LRTIs), that is they were hospitalised with the RSV LRTI (Hull et al. 2000; Hoebee et al. 2003; Hoebee et al. 2004; Janssen et al. 2007; Mailaparambil et al. 2008). Only one study (Siezen et al. 2009) has investigated prematurely born infants; two single nucleotide polymorphisms (SNPs) in IFNγ and ADAM33 were found to be associated with an increased risk of developing severe RSV infection (Siezen et al. 2009). The aim of this study was to determine if prematurely born infants may have a genetic predisposition to symptomatic RSV LRTIs regardless of whether the infants were hospitalised with the LRTI.

Prematurely born infants may be predisposed to symptomatic RSV LRTIs by abnormal premorbid lung function (Broughton et al. 2006). Amongst infants born prior to 32 weeks of gestational age, those who subsequently developed a RSV LRTI compared to controls had a higher resistance of the respiratory system (R_{rs}) at 36 weeks post menstrual age (PMA) (Broughton et al. 2006). A further aim, therefore, was to determine if any possible genetic predisposition to symptomatic RSV LRTIs was associated with reduced premorbid lung function in prematurely born infants.

RSV LRTIs in prematurely born infants are associated with chronic respiratory morbidity and lung function abnormalities at follow up (Broughton et al. 2005; Broughton et al. 2007; Greenough et al. 2009). An additional aim, therefore, was to determine if some prematurely born infants may have a genetic predisposition to those adverse outcomes.

3.2 Methods

A prospective cohort study was undertaken in which infants born less than or equal to 36 weeks of gestational age were eligible for entry if they were born prior to the onset of the RSV season in 2008 or 2009. Consecutive infants, whose parents gave informed written consent, were recruited. Blood or buccal swabs were obtained from the infants prior to maternity unit discharge. Following discharge from the neonatal or maternity unit, infants were followed prospectively until one year corrected age. The parents were asked to contact the research team when their infant was symptomatic with signs consistent with a LRTI; that is cough, wheeze, and/or shortness of breath. In addition, parents were telephoned every two weeks by researchers to ascertain whether their infant had been or was symptomatic. A researcher visited the home on each occasion that an infant had a LRTI and a nasopharyngeal aspirate (NPA) was obtained if the LRTI was confirmed. NPAs were also obtained from all infants hospitalised with an LRTI. Real time reverse transcriptase polymerase chain reaction (PCR) was performed on the NPAs for 11 virus types (influenza A and B, RSV A and B, human metapneumovirus,

rhinoviruses, parainfluenza viruses 1–3, enteroviruses and parechovirus) and real-time PCR was performed for human bocavirus and adenovirus as described in section 2.7.

The infants underwent lung function measurements at 36 weeks PMA whilst still inpatients on the neonatal or maternity unit. Functional residual capacity by helium gas dilution (FRC_{He}) and compliance (C_{rs}) and resistance (R_{rs}) of the respiratory system were measured as described in section 2.3. The infants also underwent lung function measurements at one year corrected age. FRC_{He}, C_{rs}, R_{rs}, FRC by the multiple breath wash-in/out technique (FRC_{MBW}) and lung clearance index (LCI) were assessed as described in section 2.3. FRC (FRC_{pleth}) and airway resistance (R_{aw}) by plethysmography were also assessed as described in section 2.4. Infants were assessed if they had not been symptomatic with a respiratory tract infection during the previous three weeks.

Parents completed a respiratory diary for one month when their infant was 11 months corrected age and filled in a respiratory health related questionnaire about their infant when the infant was one year of corrected age as described in section 2.6. In addition, the infants' hospital notes were reviewed and all hospitalisations in the first year after birth documented as described in section 2.5.

As part of the cohort study, blood or buccal swabs were obtained from the infants prior to maternity unit discharge. Either one mL of blood was

collected into an EDTA tube or three buccal swabs were obtained and stored at -20^{o}C until testing. The samples were then sent on dry ice to the National Institute for Public Health and the Environment (RIVM) in Bilthoven, the Netherlands for testing. DNA was isolated from the blood samples or buccal swabs as described in section 2.8. Extracted DNA samples were diluted with TE Buffer to 7 ng/µL and sent to KBioscience (Herts, UK) for genotyping to test nine SNPs (ADAM33 rs2787094, IL10 rs1800872, IL19 rs2243191, KLRG1 rs1805723, MMP16 rs2664349, MMP16 rs2664352, NFκB1A rs2233409, SFTPC rs1124 and TGFβR1 rs334353) and two other SNPs (VDR rs10735810 and NOS2A rs1060826) were tested in the Netherlands as described in section 2.8. The SNPs were chosen as they had previously been associated with an increased risk of severe RSV infection in infants born at term (Hoebee et al. 2004; Wilson et al. 2005; Janssen et al. 2007; Helminen et al. 2008) or born prematurely (Siezen et al. 2009), an increased risk of developing RDS (Lahti et al. 2002), a decreased risk of developing BPD (Hadchouel et al. 2008), an increased risk of recurrent wheeze at one year of age after RSV LRTI (Ermers et al. 2011) or reduced preschool lung function (Simpson et al. 2005). The genotype distributions of all polymorphisms described in this study were in Hardy-Weinberg equilibrium.

3.3 Analysis

A sample size of 160 for the whole prospective cohort study was planned to give 90% power at the 5% level to detect a difference between groups

in the 36 week PMA lung function results equivalent to at least one standard deviation.

The infants were divided into two groups:

(i) Infants who had at least one symptomatic LRTI from which RSV was detected from the NPA (RSV LRTI group)

(ii) Infants who did not have RSV detected from an NPA (control group)

Data were tested for normality using the Shapiro-Wilk test. Birth weight was normally distributed and differences were assessed for statistical significance using the independent student's t-test. All other variables were non-normally distributed and differences were assessed for statistical significance using either the Mann-Whitney U test, the Kruskal-wallis test, the Chi squared or Fisher's exact test. Post hoc analysis was performed on the genotype data using ANOVA if the initial analysis demonstrated a significant difference in the results. Statistical analysis was performed using IBM SPSS Statistics (version 19, New York, USA).

3.4 Results

Two hundred and fifty one infants were eligible for inclusion into the over arching study (Figure 3.1, Appendix 5). One hundred and forty eight infants completed this part of the study; they had a median gestational age of 34 (range 23-35) weeks and birth weight of 1885 (range 534-3610)

g. Forty infants (27%) were of Caucasian origin, 37 (25%) black Caribbean, 31 (21%) black African, seven (5%) Asian, three (2%) Hispanic and 30 (20%) mixed ethnicity. Fifteen (10%) infants developed BPD; that is they were oxygen dependent beyond 28 days of life. Six infants received palivizumab, one of whom was subsequently hospitalized with an RSV LRTI, but did not have lung function assessment at one year.

Twenty nine infants developed a RSV LRTI, ten required hospitalization. Compared to infants who did not develop a RSV LRTI, those that did were born at a lower gestational age (p=0.024), had a lower birth weight (p=0.038) and a smaller proportion were breastfed (p=0.042) (Table 3.1).

There was an increased risk of developing a RSV LRTI associated with the C allele of the ADAM33 gene (p=0.04), but not with any other SNPs (Table 3.2). Differences in the ADAM33 gene at the allele level between the infants admitted and not admitted to hospital for RSV LRTI and controls did not reach statistical significance (p=0.07) (Table 3.3). To determine whether the increased risk of RSV LRTI associated with the ADAM33 gene was associated with premorbid lung function, the lung function results at 36 weeks PMA were related to the ADAM33 genotype and allele distributions in all infants regardless of whether they had developed RSV LRTIs. No significant differences were found in any of the lung function results at 36 weeks PMA between the ADAM33 genotypes (Table 3.4) or allele distributions (data not shown).

There was a significant difference at the genotype level in the IL10 SNP (p=0.045) with regard to parental reported wheeze. Post hoc analysis demonstrated that significantly fewer infants with the AA genotype had parental reported wheezing than the AC genotype (p=0.045). There was a significant difference at the allele level in the IL10 SNP (AA versus AC and CC) in the proportion of infants with parental reported wheeze (17% versus 73%, p=0.022). There were significant differences at the genotype level in the ADAM33 SNP (CC versus CG versus GG) with regard to the number of acute visits to hospital and/or GP (median [range]: 0 [0] versus 1 [0-5] versus 0 [0], p=0.038), but there were no post hoc significant differences between genotypes. There were significant differences at the genotype level in the NOS2A SNP (CC versus CT versus TT) with regard to the number of days of cough (median [range]: 0 [0-13] versus 12.5 [0-30] versus 7 [0-14], p=0.028), with post hoc analysis demonstrating that the CT genotype was associated with significantly more days of cough than the CC genotype (p=0.023). There were significant differences at the allele level in the ADAM33 SNP (CC and CG versus GG), with regard to the number of parental reported acute visits to hospital and/or GP (median [range]: 0 [0] versus 0.5 [0-5], p=0.040). There were significant differences in the SFTPC SNP (AA and AG versus GG), with regard to the number of parental reported acute visits to hospital and/or GP (median [range]: 1 [0-5] versus 0 [0-1], p=0.031) and in the NOS2A SNP (CC versus CT and TT) with regard to the number of days of cough (median [range]: 0 [0-13] versus 12.5 [0-30], p=0.011).

Seventy infants had lung function measurements at one year of corrected age. There were significant differences in the lung function results at one year corrected age with regard to the MMP16 (rs2664349), MMP16 (rs2664352), NOS2A and SFTPC genes and differences with regard to the VDR gene were at borderline significance (Table 3.5).

Figure 3.1: Flow diagram of recruitment

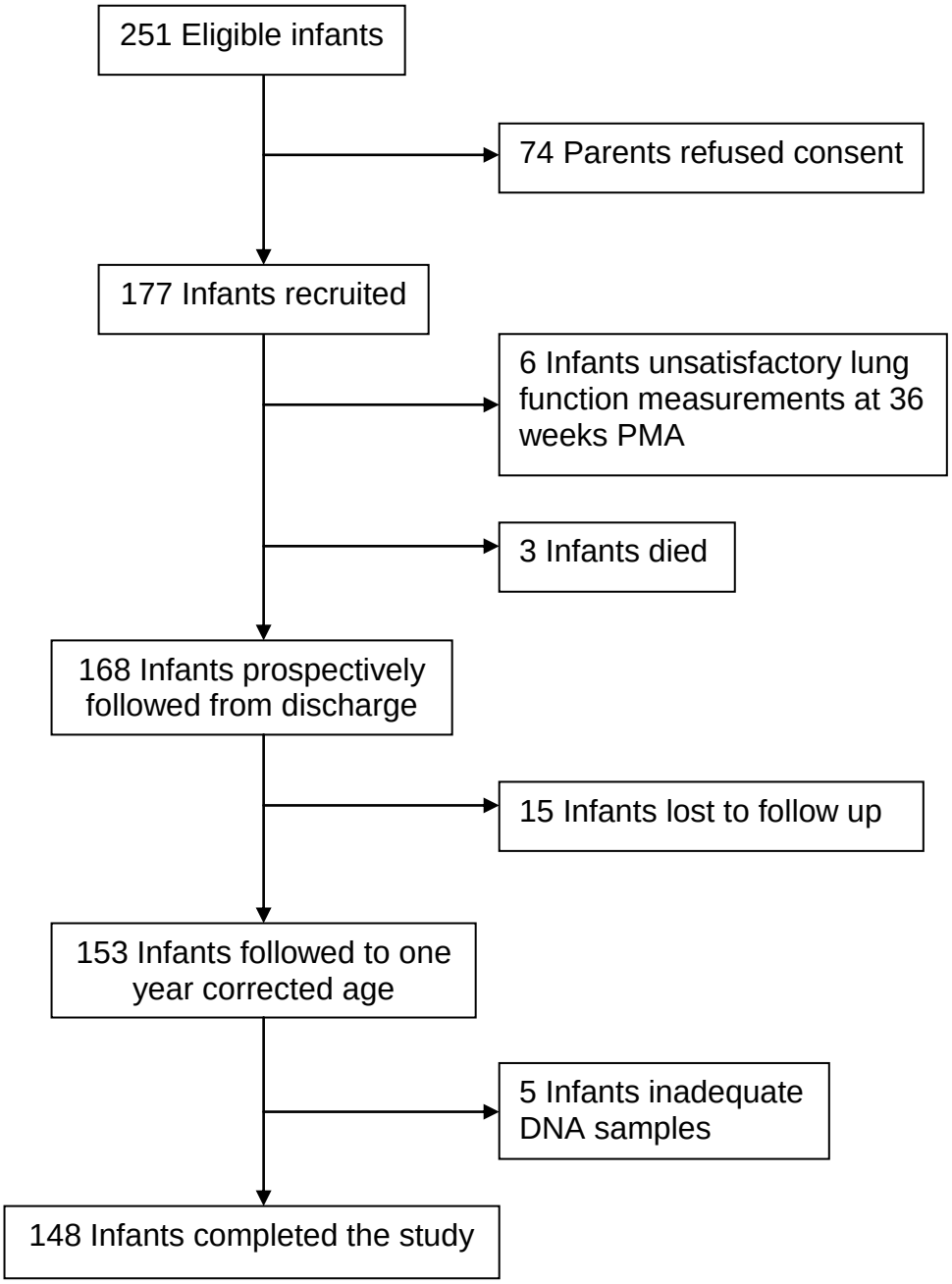


Table 3.1: Demographic data according to RSV LRTI status

Data are presented as median (range) or n (%).

	RSV LRTI	Controls	p
n	29	119	
Gestational age (weeks)	33 (23-36)	34 (24-36)	0.024
Birth weight (g)	1756 (610-2650)	1950 (534-3610)	0.038
Males	14 (48%)	67 (56%)	0.53
Antenatal smoking	4 (14%)	20 (17%)	>0.99
Antenatal steroids	24 (83%)	78 (66%)	0.078
Surfactant	7 (24%)	24 (20%)	0.62
Duration of ventilation (days)	1 (0-81)	1 (0-113)	0.34
Bronchopulmonary dysplasia	3 (10%)	12 (10%)	>0.99
Family history of atopy	15 (52%)	81 (68%)	0.13
Breastfed	19 (66%)	99 (83%)	0.042
Number of siblings	1 (0-4)	1 (0-5)	0.34
Palivizumab	1 (3%)	5 (4%)	>0.99

Table 3.2: Associations at the genotype and allele levels according to RSV status

Data are presented as n (%) or median (range)

		Association at the genotype level				Association at the allele level				
Gene	rs number	Genotype	RSV	Control	p	Allele	RSV	Control	p	OR
A disintegrin and metalloprotease 33 (ADAM33)	rs2787094	CC	4 (14%)	12 (10%)	0.038	C	25 (43%)	68 (29%)	0.04	1.89 (1.01-3.56)
		CG	17 (58%)	44 (37%)		G	33 (57%)	170 (71%)		0.53 (0.28-0.99)
		GG	8 (28%)	63 (53%)						
Pulmonary surfactant protein C (SFTPC)	rs1124	AA	0 (0%)	12 (10%)	0.21	A	7 (12%)	54 (23 %)	0.10	0.47 (0.18-1.15)
		AG	7 (24%)	30 (25%)		G	51 (88%)	184 (67%)		2.14 (0.87-5.49)
		GG	22 (76%)	77 (65%)						
IL10	rs1800872	AA	6 (21%)	10 (9%)	0.16	A	25 (45%)	75 (33%)	0.12	1.67 (0.88-3.15)
		AC	13 (46%)	55 (48%)		C	31 (55%)	155 (67%)		0.60 (0.32-1.13)
		CC	9 (33%)	50 (43%)						
Matrix metalloproteinase-16 (MMP16)	rs2664352	CC	4 (14%)	29 (25%)	0.39	C	24 (41%)	121 (51%)	0.19	0.67 (0.36-1.25)
		CT	16 (55%)	63 (53%)		T	34 (59%)	115 (49%)		1.26 (0.64-2.50)
		TT	9 (31%)	26 (22%)						
MMP16	rs2664349	GG	7 (24%)	15 (13%)	0.33	G	26 (45%)	86 (37%)	0.29	1.38 (0.74-2.57)
		AG	12 (41%)	56 (48%)		A	32 (55%)	146 (63%)		0.73 (0.39-1.35)
		AA	10 (35%)	45 (39%)						
Vitamin D receptor (VDR)	rs10735810	AA	2 (7%)	16 (13%)	0.73	A	16 (28%)	77 (32%)	0.53	0.80 (0.40-1.57)
		AG	12 (41%)	45 (38%)		G	42 (72%)	161 (68%)		1.26 (0.64-2.50)
		GG	15 (52%)	58 (49%)						
Nitric oxide synthase type 2A (NOS2A)	rs1060826	TT	2 (7%)	13 (11%)	0.32	T	18 (31%)	65 (27%)	0.63	1.20 (0.61-2.33)
		CT	14 (48%)	39 (33%)		C	40 (91%)	173 (73%)		0.84 (0.43-1.64)
		CC	13 (45%)	67 (56%)						
Nuclear factor-κ-B 1A (NFκB1A)	rs2233409	TT	1 (4%)	7 (6%)	0.74	T	10 (28%)	47 (20%)	0.85	0.87 (0.38-1.96)
		CT	8 (29%)	33 (28%)		C	46 (72%)	189 (80%)		1.14 (0.51-2.62)
		CC	19 (68%)	78 (66%)						
Killer cell lectin-like receptor subfamily G member 1 (KLRG1)	rs1805723	GG	0 (0%)	11 (9%)	0.11	G	13 (22%)	57 (24%)	0.87	0.92 (0.44-1.91)
		AG	13 (45%)	35 (29%)		A	45 (78%)	181 (76%)		1.09 (0.53-2.30)
		AA	16 (55%)	73 (62%)						
Transforming growth factor-β receptor-1 (TGFβR1)	rs334353	GG	1 (3%)	9 (8%)	0.51	G	15 (26%)	58 (25%)	0.87	1.07 (0.53-2.16)
		GT	13 (45%)	40 (34%)		T	43 (74%)	178 (75%)		0.93 (0.46-1.90)
		TT	15 (52%)	69 (58%)						

IL19	rs2243191	TT	1 (3%)	4 (3%)	>0.9	T	11 (19%)	44 (18%)	1.00	1.03 (0.46-2.26)
		CT	9 (31%)	36 (30%)	9	C	47 (81%)	194 (82%)		0.97 (0.44-2.16)
		CC	19 (66%)	79 (67%)						

Table 3.3: ADAM33 genotype and allele associations by RSV hospitalisation status

Data are presented as n (%).

			Association at the genotype level					Association at the allele level			
Gene	rs number	Genotype	RSV admitted	RSV not admitted	Control	p	Allele	RSV Admitted	RSV not admitted	Control	p
ADAM33	rs2787094	CC	1 (10%)	3 (16%)	12 (10%)	0.04	C	10 (50%)	15 (39%)	68 (38%)	0.07
		CG	8 (80%)	9 (47%)	44 (37%)		G	10 (50%)	23 (61%)	110 (62%)	
		GG	1 (10%)	7 (37%)	63 (53%)						

Table 3.4: Lung function results at 36 weeks PMA by ADAM33 genotype

Data presented as median (range).

Lung function test	Genotype	Lung function results	p value
FRC_{He} (mL/kg)	CC	26 (16-30)	0.73
	CG	26 (14-35)	
	GG	25 (8-35)	
R_{rs} (cm/H_2O/L/s)	CC	77 (51-144)	0.52
	CG	70 (43-149)	
	GG	73 (48-199)	
C_{rs} (mL/cmH_2O/kg)	CC	1.4 (0.8-2.7)	0.95
	CG	1.4 (0.7-2.4)	
	GG	1.4 (0.4-2.4)	

Table 3.5: Statistically significant genotype distributions in infants with RSV LRTI related to lung function results at one year corrected age

Data presented as median (range)

Gene	Genotype*	Lung function test	Lung function results	p
MMP16 (rs2664349)	GG (n=3)	LCI	7.3 (6.5-7.3)	0.037
	AG (n=6)		7.5 (7.1-8.2)	
	AA (n=4)		7.0 (6.7-7.1)	
MMP16 (rs2664349)	A (n=4)	LCI	7.0 (6.7-7.1)	0.031
	G (n=9)		7.3 (6.5-8.2)	
MMP16 (rs2664349)	A (n=4)	FRC_{MBW} (ml/kg)	27 (20-31)	0.045
	G (n=9)		19 (13-26)	
NOS2A	C (n=14)	C_{rs} (ml/cmH_2O/kg)	1.6 (1.2-2.5)	0.039
	T (n=2)		1.2 (1.2-1.2)	
NOS2A	C (n=14)	FRC_{MBW} (ml/kg)	21 (16-31)	0.03
	T (n=2)		14 (12-14)	
NOS2A	C (n=14)	LCI	7.1 (6.5-7.6)	0.03
	T (n=2)		8.0 (7.7-8.2)	
MMP16 (rs2664352)	C (n=9)	$FRC_{He:pleth}$	0.82 (0.68-0.97)	0.027
	G (n=5)		0.97 (0.84-0.99)	
SFTPC	A (n=4)	C_{rs} (ml/cmH_2O/kg)	1.2 (1.2-1.5)	0.025
	G (n=12)		1.6 (1.2-2.5)	
VDR	A (n=9)	C_{rs} (ml/cmH_2O/kg)	1.6 (1.2-2.5)	0.050
	G (n=7)		1.3 (1.2-1.9)	

*The number of infants with lung function results with a particular genotype, some infants did not complete all tests

3.5 Discussion

This study has demonstrated a polymorphism in the ADAM33 gene was associated with an increased risk of developing RSV LRTIs in prematurely born infants, regardless of whether hospital admission was required. Previously, it has been demonstrated that the ADAM33 gene C allele was associated with an increased risk of severe symptomatic RSV infection (Siezen et al. 2009). This study, however, did not find any significant association with premorbid lung function and the ADAM33 SNP, suggesting genetic predisposition may act via a mechanism other than abnormal premorbid lung function. The ADAM33 SNP in a population based birth cohort study of children born at term was reported to be associated with reduced specific airways resistance at five, but not three years of age (Simpson et al. 2005), although no increased risk of wheezing or a doctor diagnosis of asthma at either time point was demonstrated (Simpson et al. 2005). In this study of prematurely born infants there were significant differences at the allele level in the ADAM 33 SNP with regard to the number of acute visits to the hospital and/or GP, although not in lung function results at one year.

Despite studying a relatively small number of patients, this study demonstrated that SNPs in several genes were associated with chronic respiratory morbidity and lung function abnormalities at follow up in infants who had RSV LRTIs. The C allele in the IL10 SNP was associated with an increased risk of parental reported wheeze at one year corrected age. Two

studies of previously healthy, term born infants hospitalised for RSV infection found no association between that IL10 SNP and post-bronchiolitic wheeze at follow up at one (Schuurhof et al. 2011) and six (Ermers et al. 2007) years of age. Another study (Nuolivirta et al. 2009) investigating a different IL10 SNP also failed to find any association with post-bronchiolitic wheeze at 15 months follow up. The discrepancies with those results (Ermers et al. 2007; Nuolivirtai et al. 2009; Schuurhof et al. 2011) and the current study suggest that post-bronchiolitic wheeze may have a different genetic aetiology in infants born at term compared to those born prematurely. This study also found an increased risk of cough at one year corrected age in infants with the CC and CT genotypes of the NOS2A SNP. In addition, the NOS2A T allele was associated with significantly more days of cough and the TT genotype was associated with reduced lung function at follow up (significantly lower C_{rs}, higher LCI and lower FRC_{MBW}). SNPs in the NOS2A gene have been associated with an increased risk of developing severe RSV infection (Janssen et al. 2007), but also with reduced lung function and an increased risk of asthma in adolescent children born at term (Islam et al. 2010).

The surfactant protein C (SFTPC) SNP has been associated with an increased predisposition to RDS in prematurely born infants (Lahti et al. 2002). The A allele in the SFTPC SNP in this study was associated with reduced lung function at follow up and an increased number of acute visits to hospital or the GP amongst the infants who had RSV LRTIs, but not the development of symptomatic RSV LRTIs. SNPs in MMP16 (rs2664349 and rs2664352) have been previously associated with a reduced risk of

developing BPD in prematurely born infants (Hadchouel et al. 2008). The reduced FRC_{MBW}, increased LCI and reduced $FRC_{He:pleth}$ associated with those two SNPs in this study are lung function abnormalities compatible with previous BPD.

The minor T allele in the SNP (rs10735810) in the VDR gene has been associated with an increased risk of developing severe RSV LRTI requiring hospitalisation in term born, Caucasian Dutch and black South African infants (Janssen et al. 2007; Kresfelder et al. 2011). In addition, amongst Canadian infants less than two years of age of Caucasian or Aboriginal descent, a different VDR polymorphism (the FokI ff genotype) was associated with an increased risk of developing acute LRTIs, 82% of which were RSV positive (Roth et al. 2008). SNPs in the VDR gene have also been shown to result in an increased risk of developing asthma (Raby et al. 2004; Saadi et al. 2009) and reduced lung function in knock-out mice (Berndt et al. 2011). Although, in this study of an ethnically diverse population of prematurely born infants we did not find any significant associations of SNPs in the VDR gene with the development of RSV LRTIs, the TT genotype of the VDR SNP was associated with reduced lung function following a RSV LRTI.

This study has a number of strengths and some limitations. A cohort of ethnically diverse, prematurely born infants documenting viral LRTIs both in the community and in hospital was prospectively followed. The ethnic diversity of our population may have influenced our results as ethnic differences in genotype may increase the risk that associations occur by

chance. Amongst the infants with RSV LRTIs, there were no significant differences in ethnic origin for each of the 11 SNPs, but the numbers in each group were too small to result in a meaningful conclusion. This study, as in others, cannot comment on non symptomatic RSV infections. Chronic respiratory morbidity was assessed using both respiratory health related questionnaires and diary cards and documented hospitalisations from the infants' hospital records, as well as measuring lung function at one year corrected age in a subset of infants. Lung function testing was undertaken at 36 weeks PMA, hence assessing infants before they were exposed to RSV infection. The majority of the population were born moderately prematurely, but it has been well documented that moderately prematurely born infants can suffer severe RSV infections and adverse consequences (Sampalis 2003; Palmer et al. 2010; Shefali-Patel et al. 2012), hence the results are likely to be generalisable to all prematurely born infants. In the statistical analysis, however, there was no correction for multiple testing so it is possible certain of the results could be due to chance and hence, the results should be considered as hypothesis generating.

In conclusion, despite a relatively small study population, the results of this study suggest prematurely born infants may have a genetic predisposition to developing symptomatic RSV LRTIs which does not appear to be influenced by premorbid reduced lung function. In addition, the results suggest that in prematurely born infants those genetic mechanisms appear distinct from the genetic mechanisms underlying chronic respiratory morbidity and reduced lung function at follow up following RSV LRTIs. Further work is necessary to

determine whether certain polymorphisms could be used to inform targeted use of prophylactic strategies.

Chapter 4: Lung function prior to viral lower respiratory tract infections in prematurely born infants

4.1 Introduction

Respiratory syncytial virus (RSV) infection in previously healthy infants (Stokes et al. 1981; Hall et al. 1984; Noble et al. 1997; Korppi et al. 2004) and those born prematurely (Broughton et al. 2007) is associated with lung function abnormalities at follow up. The abnormalities include elevated thoracic gas volume and airway resistance at one year of age (Stokes et al. 1981; Broughton et al. 2007), airways obstruction in children (Hall et al. 1984; Noble et al. 1997) and lower forced expiratory volume over one second and maximal expiratory flow at 25% of forced vital capacity in young adults (Korppi et al. 2004). It is possible, however, that those lung function abnormalities at follow up may reflect that infants who develop symptomatic LRTIs have diminished premorbid lung function. In one study (Young et al. 1995), term born infants who developed bronchiolitis in the first year after birth compared to those who did not, had a non-significant trend to a lower maximal flow at functional residual capacity (V_{max}FRC) at five weeks of age, that is, before they developed bronchiolitis. In addition, in the Tucson Children's Respiratory Study, compared with controls, infants who had wheezing LRTIs in their first year had lower respiratory conductances prior to the LRTI (Martinez et al. 1988) and infants who had wheezy LRTIs in the first three years had lower premorbid V_{max} FRCs prior to the LRTI (Martinez et al. 1991). Among infants born before 32 weeks of gestation, those who developed a RSV LRTI had a significantly higher resistance of the respiratory system at 36 weeks postmenstrual age (PMA) compared to those who did not (Broughton et al. 2006). The symptomatic "RSV LRTI" group, however,

had similar resistance results to the “RSV negative LRTI” group (Broughton et al. 2006), perhaps suggesting that diminished lung function in prematurely born infants might predispose to other viral LRTIs, as well as RSV LRTI, but the numbers of infants included in the study was small (n=39) (Broughton et al. 2006). The aim of this study, therefore, was to test in a larger cohort, the hypothesis that diminished premorbid lung function would predispose prematurely born infants not only to symptomatic RSV LRTIs, but also other viral LRTIs. In addition, the study aimed to determine whether infants who developed severe LRTIs, that is, they required hospitalisation, had poorer lung function than those who did not require hospitalisation for the LRTI. The results of such a study would be important to determine whether assessment of lung function might be useful to identify a high risk group who could be targeted for prophylaxis against viral infections. As a consequence, this study related premorbid lung function in prematurely born infants to the occurrence of LRTIs. Infants with a wide range of gestational ages have been studied, so additionally it was possible to determine whether maturity at birth influenced any functional predisposition to viral LRTIs.

4.2 Methods

Infants born at less than 36 weeks of gestational age were eligible for entry into the study if they were born prior to the onset of the RSV season in 2008 or 2009. The RSV season was defined as 1 October to 31 March, consistent with the UK experience (Clark 2000). Consecutive infants, whose parents gave informed written consent, were recruited. Lung function (FRC_{He},

FRC_{MBW}, LCI, C_{rs} and R_{rs}) was performed at 36 weeks PMA as described in section 2.3. The mean intrasubject coefficient of variation for FRC_{He} was 5%, C_{rs} 12%, R_{rs} 11%, FRC_{MBW} 16% and LCI 13%. Following neonatal unit discharge, infants were followed prospectively as described in section 2.1, during their first RSV season. For this study, real time reverse transcriptase polymerase chain reaction (PCR) was performed on the NPAs for nine viruses (RSV A and B, human metapneumovirus, rhinovirus, influenza A and B, parainfluenza 1-3) in three multiplexes with a monoplex varicella zoster virus (VZV) RNA internal control as described in section 2.7. Adenovirus was tested by real-time PCR in monoplex with the VZV internal control as described in section 2.7.

4.3 Analysis

The infants were divided into four groups:

(i) Infants who never had a symptomatic LRTI - no LRTI.

(ii) Infants who had symptomatic LRTI(s), but no viruses were detected from the NPA - viral negative LRTI.

(iii) Infants who had at least one LRTI, from which RSV was detected from the NPA - RSV LRTI

(iv) Infants who had symptomatic LRTI(s) with viruses other than RSV detected from the NPA - other viral LRTI.

Data were tested for normality using the Shapiro-Wilk test. Birth weight and FRC results were normally distributed and differences were assessed for

statistical significance using the one way analysis of variance (ANOVA), all the other results were not normally distributed, hence difference were assessed for statistical significance using the Kruskal-Wallis test. Multiple comparisons were performed using a one way ANOVA with a post hoc Bonferroni test or the Kruskal-Wallis test with a post hoc Dunn's test. The relationship between R_{rs} and the occurrence of either RSV LRTI or other viral LRTI correcting for gestational age was assessed using analysis of covariance. The threshold for significance was $p<0.05$. To give an estimate of the value of lung function as a predictor of subsequent severe RSV LRTI (i.e. hospitalisation) areas under receiver operating characteristic curves were calculated and compared using SPSS (version 19, New York, USA) for those factors which differed significantly between infants with RSV LRTI who were and were not hospitalised. Other statistical analysis was performed using GraphPad Prism (version 5, La Jolla, CA, USA).

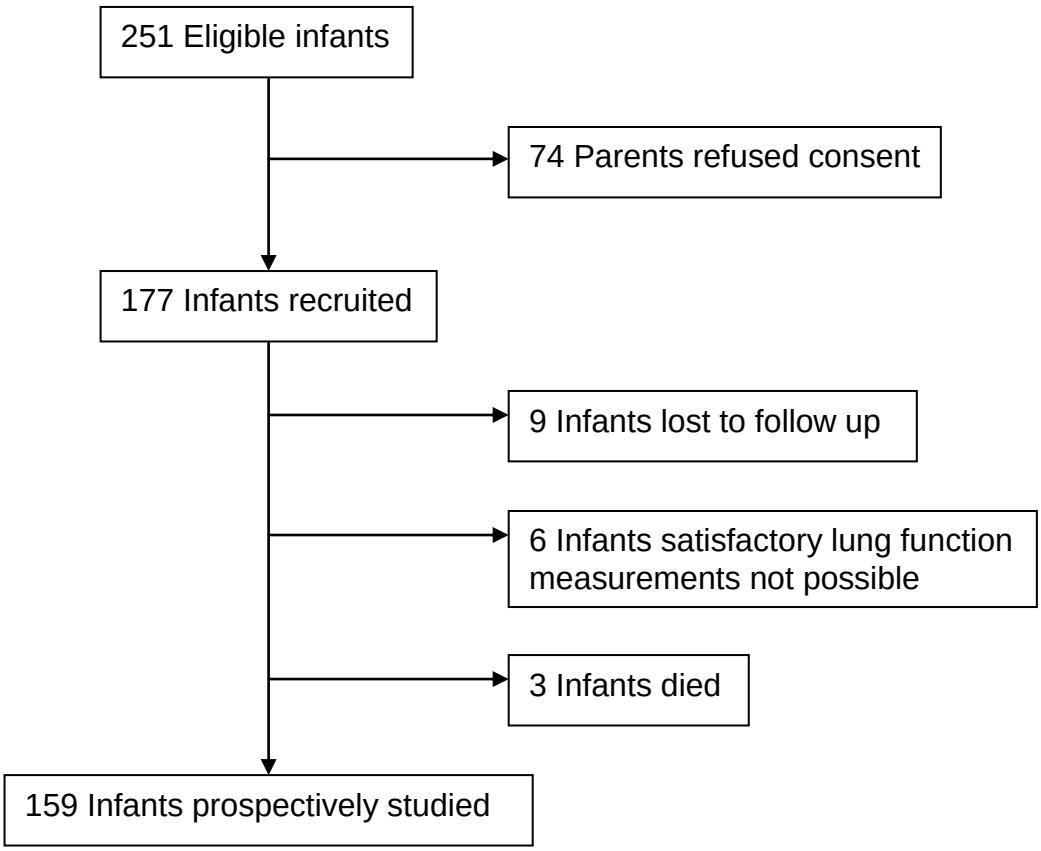
4.4 Sample size

In a previous study (Broughton et al. 2006) it was found there was a difference in the lung function results equivalent to one standard deviation (SD) between the groups. The planned sample size, therefore, was 160 infants to ensure there were at least 20 infants in each of the RSV and other viral LRTI groups to give 90% power at the 5% level to detect a difference in the lung function results equivalent to at least one standard deviation between each of those two groups and the no LRTI group.

4.5 Results

Two hundred and fifty one infants were eligible for inclusion into the study (Figure 4.1, Appendix 5).

Figure 4.1: Flow diagram of recruitment



Seventy four parents did not consent to their infant taking part in the study and nine parents consented, but then defaulted from follow up. One infant had a tracheostomy and lung function measurements were not possible and five infants had unsatisfactory lung function recordings. One infant died before discharge and two infants died shortly after discharge. The 159 (88

males) infants who completed the study had a median GA of 34 (interquartile range [IQR] 31-35) weeks and a median birth weight of 1890 (IQR 1446-2308) g. The infants who completed the study were of lower GA (median GA 34 versus 35 weeks, p=0.03) and birth weight (median birth weight 1890 versus 2132 g, p=0.08) than those who did not take part. No other data was collected from infants who were not recruited into the study. Overall, 32 (20%) mothers had had an antenatal infection and 110 (69%) had received antenatal steroids. Thirty two (20%) infants had received surfactant, 55 (35%) infants had had a postnatal infection and 16 (10%) developed BPD. Seven infants received palivizumab (Table 4.1). One of the seven infants who received palivizumab was admitted to hospital and that was because of a RSV LRTI. Two infants had a nosocomial infection due to rhinovirus while on the neonatal unit, in both cases this was after lung function testing. One of the infants subsequently developed a RSV LRTI and their results were analysed in the RSV group; the other did not subsequently have an RSV infection and their results were analysed in the other viral LRTI group.

The only significant differences in the demographics of the four groups were with regard to birth weight (p=0.02) and the number of infants receiving surfactant (p=0.03) (Table 4.1). Those differences were due to the other viral LRTI group being of lower birth weight ($p<0.05$) and a greater proportion of them having received surfactant ($p<0.05$) than the no LRTI group.

On 138 occasions, 73 infants had a LRTI and on 94 occasions a virus was detected (Table 4.2). Twenty seven infants had at least one RSV positive

LRTI (some also had other viral LRTIs), eleven required hospital admission (nine for RSV LRTI [one of those infants required intensive care] and two for non-respiratory causes). The infants who were admitted compared to those who were not were born at an earlier GA (median, [IQR] 30 [29-33] versus 34 [33-35] weeks, p=0.004), were of lower birth weight (1446 [1020-1815] versus 1852 [1515-2100] g, p=0.047), had required more days of ventilation on the neonatal unit (6 [1.3-220] versus 0 [0-1], p=0.011) and more days of supplementary oxygen on the neonatal unit (7 [1-49] versus 0 [0-1] days, p=0.011) and a greater proportion had had BPD (33% versus 0%, p=0.013). They, however, had not had more NPAs taken (p=0.22). The age at LRTI presentation also did not differ significantly between those who were and were not admitted, the mean corrected age in both groups being 4.2 months (p=0.91).

Thirty one infants suffered at least one other viral LRTI, but not a RSV LRTI; three required hospital admission (two for a LRTI and one for a non-respiratory cause). There were no significant differences between those who were and were not admitted with regard to GA at birth, birth weight, duration of ventilation and supplementary oxygen on the neonatal unit, BPD, number of NPAs taken and age at LRTI presentation. Fifteen infants had LRTIs with no virus identified, four required hospital admission (two for LRTI and two for non-respiratory causes). Eighty six infants did not have a LRTI and four required hospital admission (all for non-respiratory causes). Eleven infants had at least one dual infection and two had a triple infection (RSV A/human metapneumovirus/rhinovirus and RSV B/parainfluenza 1/adenovirus). The

infant with the triple infection due to RSV A/human metapneumovirus/rhinovirus was the only infant who was admitted to an intensive care unit.

The only significant differences between the lung function results of the four groups (Table 4.3 and Figure 4.2), even after correcting for GA, were the uncorrected C_{rs} results (p=0.031), but post hoc testing demonstrated no significant differences between the groups. The infants who were admitted to hospital with a RSV LRTI compared to those who were not had higher R_{rs} results (median 88 [IQR 76-100] cmH_2O/L/sec versus median 70 [IQR 63-76] cmH_2O/L/sec) (p=0.033) and lower median uncorrected C_{rs} (2.4 [IQR 1.9-2.8] versus 3.0 [IQR 2.2-3.6] mL/cmH_2O, p=0.027), but the FRC results were similar (p=0.13). Calculation of the areas under the receiver operating characteristic curves (AUC) regarding hospitalisation for RSV LRTI revealed: gestational age AUC=0.829 (p=0.005), birth weight AUC=0.718 (p=0.063), days in oxygen AUC=0.791 (p=0.13), BPD AUC=0.650 (p=0.20), R_{rs} AUC=0.735 (p=0.045) and uncorrected C_{rs} AUC=0.756 (p=0.029). There were no significant differences between the AUCs for gestational age and R_{rs} (p=0.50) or for gestational age and uncorrected C_{rs} (p=0.55) The infants who were admitted to hospital with another viral LRTI, compared to those who were not, had higher R_{rs} results (only two infants were admitted, their R_{rs} results were 103 and 129 cmH_2O/L/sec versus median 80 [IQR 66-92] cmH_2O/L/sec [p=0.039]) and tended to have lower uncorrected compliance results (1.6 and 1.9) mL/cmH_2O versus 2.8 [IQR 2.4-3.7] mL/cmH_2O, p=0.052, but the FRC results were similar (p=0.67).

Figure 4.2: Scatter plot showing the resistance of the respiratory system results related to viral LRTI status

Individual data are shown. The horizontal lines represent the medians of each group. There are no significant differences between the four groups.

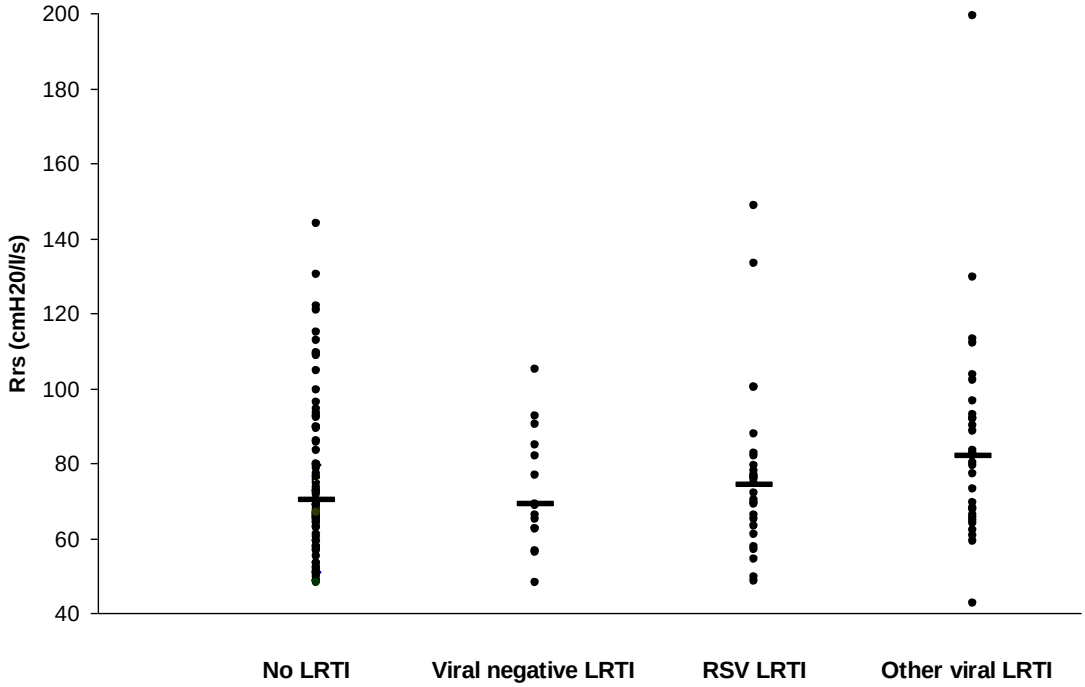


Table 4.1: Demographic data

Data are expressed as median (interquartile range) or n (%)

	No LRTI	Viral negative LRTI	RSV LRTI	Other viral LRTI	p
N	86	15	27	31	
Gestational age (weeks)	34 (32-35)	34 (31-35)	33 (30-34)	33 (29-35)	0.11
Birth weight (g)	2070 (1605-2413)	2026 (1586-2312)	1830 (1335-2065)	1565 (1261-2152)	0.02
Males	46 (56%)	9 (60%)	14 (52%)	17 (55%)	0.97
Antenatal smoking	14 (16%)	2 (13%)	4 (15%)	7 (23%)	0.82
Antenatal steroids	52 (60%)	11 (73%)	22 (81%)	25 (81%)	0.07
Surfactant	12 (14%)	2 (13%)	6 (22%)	12 (39%)	0.03
Duration of ventilation (days)	0 (0-2)	1 (0-1.5)	1 (0-5)	1 (0-4.5)	0.25
Bronchopulmonary dysplasia	6 (7%)	1 (7%)	3 (11%)	6 (19%)	0.25
Parental atopy	43 (50%)	6 (40%)	9 (33%)	12 (42%)	0.42
Day care	2 (2%)	0 (0%)	1 (4%)	2 (6%)	0.61
Number of siblings	1 (0-2)	2 (1-2)	1 (0-2)	1 (0-2)	0.17
Palivizumab	1 (1%)	1 (7%)	1 (4%)	4 (13%)	0.05

Table 4.2: Number of viruses detected by PCR

Data are displayed as the number of occasions a virus was detected.

Infants may have had more than one LRTI.

Viruses	RSV LRTI group	Other viral LRTI group
RSV A	8	0
RSV B	19	0
Rhinovirus	8	21
Adenovirus	4	8
Human Metapneumovirus	1	5
Influenza A	1	3
Influenza B	1	2
Parainfluenza 1	3	3
Parainfluenza 2	0	1
Parainfluenza 3	0	6
LRTIs for which no virus was detected	4	12

Table 4.3: Lung function results

Data are expressed as median (interquartile range).

	No LRTI (n=86)	Viral negative LRTI (n=15)	RSV LRTI (n=27)	Other viral LRTI (n=31)	p
Postmenstrual age (wks)	35.9 (35.3-36.5)	36.1 (35.7-37.1)	35.9 (35.3-36.6)	36.1 (35.6-37.5)	0.16
Weight (g)	2115 (1871-2418)	2178 (1978-2477)	1972 (1830-2170)	1970 (1829-2336)	0.10
FRC_{He} (mL/kg)	26 (26-28)	25 (22-27)	24 (21-28)	24 (22-29)	0.54
FRC_{He} (mL)	58 (48-65)	56 (47-63)	52 (42-61)	51 (43-55)	0.10
FRC_{MBW} (mL/kg)	27 (23-32)	24 (22-27)	25 (21-28)	25 (21-29)	0.63
FRC_{MBW} (mL)	56 (47-66)	59 (50-68)	52 (44-60)	52 (44-60)	0.12
LCI	9.8 (6.5-13.6)	10.0 (7.1-14.8)	9.8 (8.2-13.3)	10.2 (6.0-13.8)	0.61
C_{rs} ($mL/cmH_2O/kg$)	1.55 (1.31-1.71)	1.47 (1.19-1.53)	1.35 (1.08-1.59)	1.23 (1.08-1.73)	0.11
C_{rs} (mL/cmH_2O)	3.2 (2.6-3.6)	3.4 (2.5-3.9)	2.8 (2.1-3.6)	2.9 (2.2-3.2)	0.031
R_{rs} ($cmH_2O/L/s$)	70 (60-84)	69 (62-83)	74 (64-81)	82 (67-92)	0.12

4.6 Discussion

Infants who developed RSV or other viral LRTIs and were subsequently hospitalised had significantly poorer lung function at 36 weeks PMA than those who developed LRTIs but were not hospitalised. There were, however, no overall significant differences in the lung function results between infants who developed a RSV or other viral LRTI and those who had no LRTI in the RSV season. Those results might appear to conflict with those in the literature (Martinez et al. 1988; Young et al. 1995; Broughton et al. 2006) (Table 4.4). In the study by Young et al, however, virology results were only available for the two infants who were admitted to hospital, both were RSV positive; the other 15 infants who were not admitted had a doctor diagnosis of bronchiolitis (Young et al. 1995). There were no significant differences in the R_{rs} or C_{rs} results between their groups and there was only a non-significant trend for those who subsequently developed bronchiolitis to have a lower V_{max} FRC than those who did not (p=0.06). In another study of term born infants (Martinez et al. 1988), no virological results were reported and comparison was made between infants who did and did not subsequently develop a wheezing respiratory illness. The risk of having a wheezing illness was 3.7 times higher among infants whose values for total respiratory conductance were in the lowest third, but this was not statistically significant (p=0.06) (Martinez et al. 1988). Those results (Martinez et al. 1988) could be interpreted as indicating that diminished lung function predicts those who wheeze with a LRTI rather than those predisposed per se to a viral LRTI. In a very prematurely born group, it has been demonstrated that R_{rs} was

significantly higher in those who subsequently developed a RSV LRTI compared to the rest of the cohort (Broughton et al. 2006). The infants included in that study had worse lung function than those presently examined, reflecting a greater proportion had had bronchopulmonary dysplasia; six of the 15 infants with RSV LRTI had required hospital admission. This study, which included infants with a broader range of gestational ages, highlights R_{rs} results to be significantly higher in those infants who were admitted to hospital with a RSV or another viral LRTI, that is, they were likely to have had more severe infection.

Infants with RSV or other viral LRTIs who were hospitalised compared to those who were not had significantly worse premorbid lung function, that is higher R_{rs} results and significantly lower C_{rs} results (not corrected for body weight). In the RSV LRTI group, those who were admitted to hospital compared to those who were not were born at a significantly earlier gestational age and of lower birth weight and required significantly longer durations of ventilation and supplementary oxygen and a significantly greater proportion had developed BPD. It is, therefore, not surprising that they had significantly worse lung function at 36 weeks PMA. Comparison of the areas under the ROCs demonstrated no significant differences in the AUCs for gestational age and either R_{rs} or C_{rs} i.e. they performed similarly. This study thus suggests lung function testing at 36 weeks PMA may not only indicate a group at highest risk, but also indicates a mechanism by which prematurely born infants develop severe RSV LRTIs, that is, they are more likely to

develop a severe RSV LRTI if they have significantly poorer premorbid lung function.

Lung function was assessed by measuring C_{rs}, R_{rs}, FRC_{He}, FRC_{MBW} and LCI. The FRC and C_{rs} results were corrected for body weight, as length can be difficult to measure accurately in small infants. In the 'thinnest' infants, FRC and C_{rs} may be overestimated when a weight correction is used compared to length. For the uncorrected data the median C_{rs}, but not FRC results differed significantly in the RSV group between those who were and were not admitted, further emphasising they had worse lung function. In addition, there was a non-significant trend in corrected C_{rs} between the four groups. A possible explanation is that infants with poorer lung function may be more likely to be symptomatic with a LRTI. R_{rs} and LCI results are not routinely corrected for either body weight or length. R_{rs} results differed significantly in both the RSV and the other viral groups between those who were and were not admitted but there were no significant differences between the groups in the LCI results. There was a wide range of results reflecting the wide spread of gestational ages of the infants examined. The low intra-observer coefficients of variability in FRC_{He}, C_{rs} and R_{rs} demonstrate these results to be reliable. There were, however, much higher intra-observer coefficients of variability for the FRC_{MBW} and LCI results suggesting those data are less reliable.

The study has a number of strengths. A large cohort of infants was prospectively followed and thus the study had sufficient numbers to be able

to appropriately compare results from infants with RSV LRTI or other viral LRTI to those with no LRTI. The infants, however, were only prospectively followed during the RSV season and it is possible the infants suffered other viral infections subsequently. In addition, only two infants were admitted to hospital with other viral LRTI and thus it would be important to determine whether our results are replicated in a larger number of infants admitted to hospital with 'other' respiratory viral infections. PCR techniques were used that could detect ten respiratory viruses. The PCR techniques are more sensitive than conventional virus isolation or immunoflourescence techniques for the diagnosis of respiratory virus infections in children (Kuypers et al. 2006; van de Pol et al. 2007; Bonroy et al. 2007). A further strength of the study is that parents were asked to contact the research team whenever their infant was symptomatic and, in addition, they were reminded by two weekly telephone calls. As a consequence, it was possible to document LRTIs not only in hospital but also in the community.

In conclusion, these results suggest that, overall, prematurely born infants are not predisposed by diminished premorbid lung function to RSV or other viral LRTIs, but rather it predisposes them to hospitalisation if they develop a viral LRTI.

Table 4.4 Previous studies reporting lung function prior to lower respiratory tract infections in infancy

Data are demonstrated as mean (SD).

Reference	Sample size	GA at birth	PMA at assessment (weeks)	Viral identification	Lung Function test results			
(Young et al. 1995)	253	Term	45	RSV (n=2)		Bronchiolitis (n=10)	Control (n=236)	p value
					T_{me}/T_E (%)	30.4 (3.5)	33.7 (0.10)	NS
					R_{rs} (cmH_2O/L/s)	60 (5)	56 (10)	NS
					C_{rs} (mL/cmH_2O/kg)	1.4 (0.1)	1.4 (0.03)	NS
(Martinez et al. 1988)	124	Term	48	Virology not reported		LRTI with wheeze (n=24)	No LRTI (n=88)	p value
					T_{me}/T_E (%)	25.4 (6.9)	31.2 (9.2)	0.01
					Conductance (L/s/cmH_2O)	0.028 (0.006)	0.035 (0.009)	0.04
					FRC_{He} (mL)	97.1 (20.8)	103.2 (16.7)	0.63
					$V_{max}FRC$ (mL/s)	118.6 (51.2)	131.2 (47.9)	0.50
(Broughton et al. 2006)	39	28	36	RSV (n=15)		RSV LRTI (n=15)	No RSV LRTI (n=24)	p value
					R_{rs} (cmH_2O/L/s)	126 (62)	89 (28)	0.01
					C_{rs} (mL/cmH_2O/kg)	1.0 (0.5)	1.0 (0.5)	0.82
					FRC_{He} (mL/kg)	22 (4)	22 (3)	0.94

Key: NS= Non-significant; GA= Gestational age

Chapter 5: Lung function of preterm infants before and after respiratory viral infections

5.1 Introduction

Prematurely born children frequently suffer chronic respiratory morbidity at follow up; frequent troublesome symptoms requiring treatment and lung function abnormalities even into adolescence and young adulthood (Ali & Greenough 2012). It is possible that viral infections may contribute to that increased morbidity, as greater healthcare utilisation and lung function abnormalities at school age have been reported following respiratory syncytial virus (RSV) lower respiratory tract infections (LRTI) (Greenough et al. 2009). Poorer lung function at 36 weeks postmenstrual age (PMA), however, was found in very prematurely born infants who developed an RSV LRTI (Broughton et al. 2006). It may then be that RSV LRTIs occur in those destined to have reduced lung function at follow up because of poorer premorbid lung function. Indeed, term born infants who developed viral bronchiolitis in infancy had reduced lung function soon after birth, that is before they had had viral bronchiolitis, and similar levels of reduced lung function at 11 years of age (Turner et al. 2002). The aim of this study was to determine whether viral LRTIs affect prematurely born infants' lung function at follow up, by undertaking lung function measurements at one year corrected age.

5.2 Methods

Infants born at less than 36 weeks of gestational age (GA) were eligible for entry into the study if they were born prior to the onset of the RSV

season in either 2008 or 2009. The RSV season was defined as 1st October to 31st March, consistent with UK experience (Clark 2000). After discharge from the neonatal/maternity unit infants were prospectively followed until one year corrected age as described in section 2.1. NPAs were tested for 13 viruses by real time PCR as described in section 2.7. The infants underwent lung function measurements (FRC_{He}, FRC_{MBW}, LCI, C_{rs} and R_{rs}) at 36 weeks postmenstrual age (PMA) prior to neonatal or maternity unit discharge as described in section 2.3. Infants also underwent lung function assessment (FRC_{pleth}, R_{aw}, FRC_{He}, FRC_{MBW}, LCI, C_{rs} and R_{rs}) at one year of corrected age as described in section 2.4.

5.3 Analysis

Infants who had a LRTI but no virus was detected from the NPA were excluded from the analysis.

The remaining infants were divided into two groups:

(i) Infants who never had a symptomatic LRTI (no LRTI group)

(ii) Infants who had at least one symptomatic LRTI from which either RSV or another respiratory virus was detected from the NPA (viral LRTI group).

For continuous variables, the t-test and Mann-Whitney U test were used as appropriate to explore any differences between the two groups. Chi-squared and Fisher's exact test were used as appropriate for binary

variables using IBM SPSS Statistics (version 19, New York, USA). Multivariable regression models were developed by Professor Janet Peacock and Jessica Lo using Stata (v.12.1 TX, USA). They were used to examine the association between viral status and lung function at one year corrected age after adjusting for possible confounders and after checking that the model residuals were approximately normally distributed. Principal component analysis was used to summarise the confounder variables and provide parsimonious regression models. Neonatal factors only were adjusted for in the first stage and both infant and neonatal factors were adjusted for in the second stage. For details regarding the variables used, see footnote under Table 5.4.

5.4 Sample size

A sample size of at least 30 in each of the two groups allowed the detection of a difference in the means of the lung function test results of 0.85 standard deviations (SDs), with 90% power and two-sided 5% significance. In a previous study (Broughton et al. 2007), a difference of one SD was detected between the lung function results of infants who had or had not had a RSV LRTI.

5.5 Results

Two hundred and fifty one infants were eligible for inclusion into the study and 70 infants were included in this part of study (Figure 5.1, Appendix

5). Three (4%) infants received palivizumab, none of whom were admitted to hospital because of a RSV LRTI. Thirty-eight infants did not have a symptomatic LRTI (no LRTI group). Thirty-two infants had a viral LRTI (Table 5.1). Fourteen infants had a least one RSV LRTI but the majority of them also had another viral LRTI (rhinovirus, adenovirus, human metapneumovirus or enterovirus). Twelve infants had a dual viral LRTI and three a triple viral LRTI. Five infants in the no LRTI group and six infants in the viral LRTI group (RSV n=5) were admitted to hospital. There were significant differences between the two groups with regard to their birth weight (p=0.006), exposure to antenatal steroids (p=0.036), duration of supplemental oxygen (p=0.023) and the duration (p=0.009) and cost of neonatal unit stay (p=0.009) (Table 5.2).

There were no significant differences in the lung function results of the two groups at 36 weeks PMA (Table 5.3). At one year corrected age, the R_{rs} (p=0.024) and the R_{aw} results (p=0.0068) differed significantly between the two groups (Table 5.3). After full adjustment, the difference for R_{rs} of 6.9 $cmH_2O/L/sec$ was reduced to 5.2 $cmH_2O/L/sec$ and was no longer statistically significant (Table 5.4). The difference in the R_{aw} results between the groups was reduced from 6.0 to 4.69 $cmH_2O/L/sec$, but remained statistically significant (p=0.021) (Table 5.4). The median (range) time between between the last viral LRTI and lung function testing in infants who developed a viral LRTI was 6 (1-15) months. There were no significant correlations between the time between the occurrence

of the viral LRTI and the lung function test and any of the lung function test results at one year corrected age.

Figure 5.1: Flow diagram of recruitment

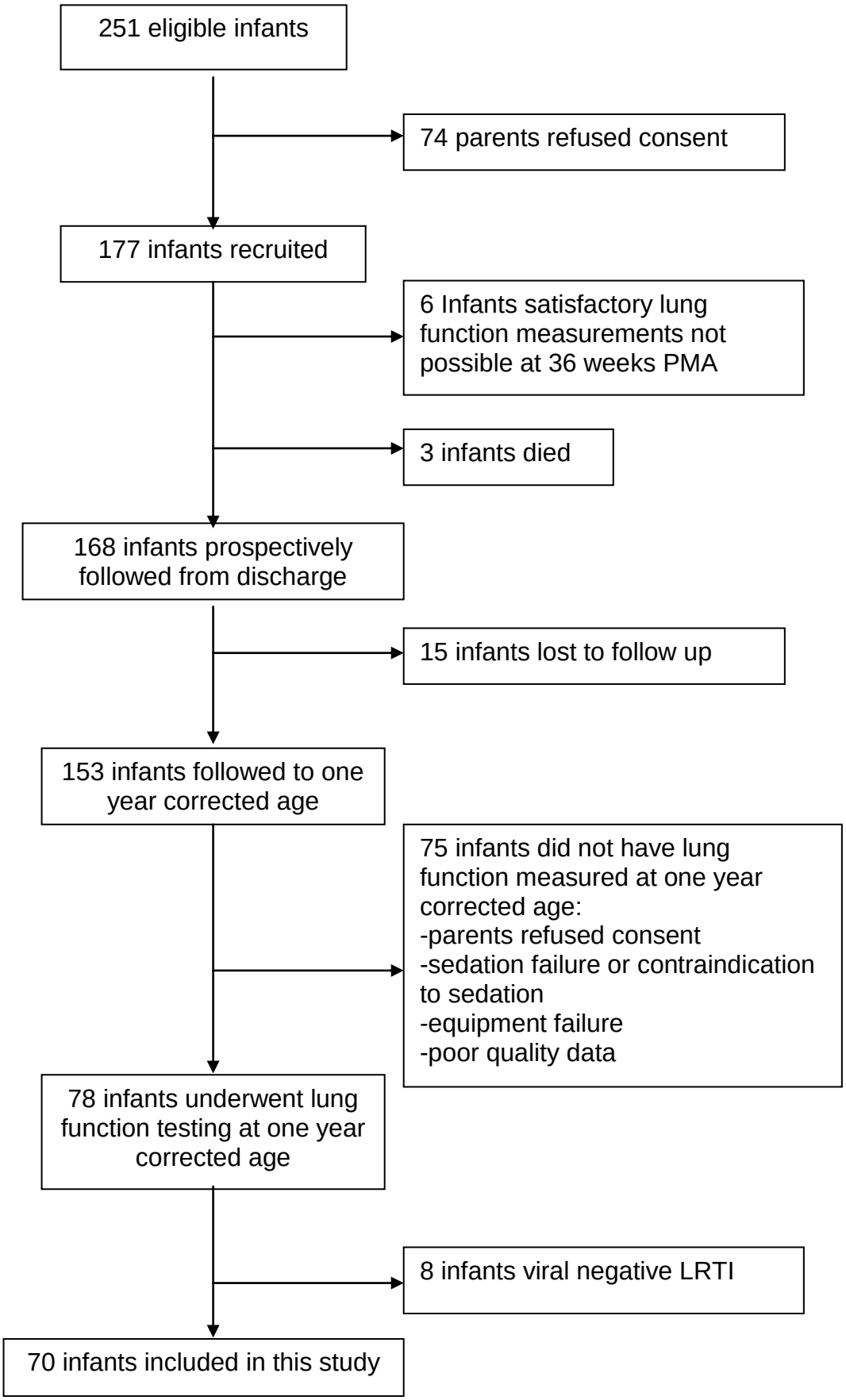


Table 5.1: Number of viruses detected by real time PCR

Data are displayed as the number of occasions a virus was detected.

Some infants may have had more than one viral LRTI.

	Viruses detected
RSV A	5
RSV B	11
Rhinovirus	9
Adenovirus	7
Human Metapneumovirus	4
Influenza A	3
Influenza B	2
Parainfluenza 1	3
Parainfluenza 2	1
Parainfluenza 3	5
Enterovirus	8
Parechovirus	0
Bocavirus	1

Table 5.2: Infant characteristics by viral LRTI status

Data are displayed as median (range), mean [SD] or n (%).

	No LRTI group	Viral LRTI group	p
N	38	32	
Gestational age (weeks)	34 (28–35)	32 (24–35)	0.17
Born < 32 weeks of gestational age	8 (21%)	13 (41%)	0.075
Birth weight (g)	2098 (900–3174)	1667 (670–3154)	0.006
Small for gestational age	4 (11%)	7 (22%)	0.32
Male	22 (58%)	17 (53%)	0.69
Antenatal smoking	5 (13%)	9 (28%)	0.14
Antenatal steroids	22 (58%)	26 (81%)	0.036
Surfactant	6 (16%)	11 (34%)	0.071
Duration of ventilation (days)	0 (0–15)	2 (0–90)	0.036
Duration of supplemental oxygen (days)	0 (0–58)	2 (0–377)	0.023
Bronchopulmonary dysplasia	3 (8%)	7 (22%)	0.17
Family history of atopy*	26 (68%)	21 (60%)	0.80
Family history of asthma	5 (16%)	9 (24%)	0.78
Number of siblings	1 (0–5)	1 (0–5)	0.39
Breastfed	35 (92%)	25 (78%)	0.17
Palivizumab given	0 (0%)	3 (9%)	0.091
Duration of neonatal unit stay (days)	18 (2–117)	29 (3–188)	0.009
Cost of neonatal unit stay (UK £)	11831 [14400]	24262 [31524]	0.009

*atopy = asthma, eczema and/or hayfever

Table 5.3: Lung function results at 36 weeks PMA and one year corrected age by virus status

Data are displayed as mean (SD) [range].

	No LRTI group	Viral LRTI group	p
n	38	32	
At 36 weeks PMA			
Postmenstrual age (weeks)	35 (1.2) [34-41]	36 (1.6) [34–41]	0.018
Weight (g)	2176 (373) [1598–3274]	2067 (377) [1200–3154]	0.23
FRC_{He} (mL/Kg)	25.5 (3.5) [19–34]	23.7 (6.2) [7.9–35]	0.12
FRC_{MBW} (mL/Kg)	26.5 (26.6) [19-42]	25.1 (25.0) [10-35]	0.24
LCI	9.7 (9.9) [6.6-13.4]	10.0 (9.7) [6.0-14.1]	0.21
C_{rs} (mL/cmH_2O/Kg)	1.48 (0.36) [0.72–2.3]	1.39 (0.39) [0.72–2.4]	0.23
R_{rs} (cmH_2O/L/s)	72.5 (20) [48–122]	79.4 (21) [43–133]	0.11
At one year corrected age			
Corrected age (months)	12.7 (2.1) [5.9–16]	13.2 (2.3) [5.4–19]	0.70
Days between assessments	416 (64) [215–514]	424 (72) [199–618]	0.93
Weight (kg)	9.9 (1.7) [6.4–16]	9.5 (1.3) [7.2–12]	0.48
Length (cm)	78 (3.3) [72–85]	78 (4.1) [71–87]	0.75
FRC_{He} (mL/kg)	24.6 (3.9) [17–32]	24.9 (3.7) [18–33]	0.81
FRC_{MBW} (mL/Kg)	21.1 (20.7) [13-32]	21.7 (21.2) [13-31]	0.97
LCI	7.3 (7.3) [6.2-9.5]	7.1 (7.1) [5.5-8.2]	0.70
C_{rs} (mL/cmH_2O/kg)	1.87 (0.72) [0.25–4.0]	1.78 (0.45) [1.2–2.7]	0.71
R_{rs} (cmH_2O/L/s)	43.4 (9.9) [28–67]	50.3 (13.9) [34–89]	0.024
FRC_{pleth} (mL/kg)	26.1 (4.3) [19–36]	27.9 (6.5) [20–48]	0.41
R_{aw} (cmH_2O/L/s)	17.2 (5.5) [9.7–33]	23.2 (9.5) [11–45]	0.0068
$FRC_{He:pleth}$ ratio	0.88 (0.07) [0.74–1.0]	0.87 (0.09) [0.68–1.0]	0.73

Table 5.4: Difference in lung function between groups (viral LRTI and no LRTI) after regression modelling to adjust for confounding neonatal and infant factors

The results are expressed as the mean (95% CI).

	Difference in lung function between viral groups after adjusting for neonatal factors*	p	Difference in lung function between viral groups after adjusting for neonatal and infant factors*	p
FRC (mL/kg)	0.52 (-1.52, 2.55)	0.61	0.28 (-1.81, 2.36)	0.79
R_{rs} (cmH_2O/L/s)	5.84 (-0.11, 11.8)	0.054	5.21 (-0.86, 11.3)	0.091
C_{rs} (mL/cmH_2O/kg)	-0.026 (-0.32, 0.27)	0.86	-0.031 (-0.34, 0.28)	0.84
FRC_{pleth} (mL/kg)	1.59 (-1.36, 4.54)	0.29	1.43 (-1.49, 4.34)	0.33
R_{aw} (cmH_2O/L/s)	5.50 (1.31, 9.68)	0.011	4.69 (0.74, 8.63)	0.021
$FRC_{He:pleth}$ ratio	-0.01 (-0.05, 0.03)	0.58	-0.01 (-0.05, 0.03)	0.69

* Neonatal factors were: gender, gestational age, birth weight, exposure to antenatal steroids and use of surfactant. Principal component analysis reduced these 5 factors to 3 components. Infant factors were: days of ventilation, BPD status, breastfed, palivizumab given and duration of stay in hospital. Principal component analysis reduced the 6 factors to 2 components.

5.6 Discussion

This study has demonstrated that, even after adjustment, the viral LRTI compared to the no LRTI group had significantly worse lung function at one year corrected, that is, after they had had viral LRTIs. There were, however, no significant differences in the lung function at 36 weeks PMA, that is, before the viral LRTIs. Those results then suggest the viral LRTIs were responsible for the deterioration in lung function. Higher airway resistance results have previously been reported at one year corrected following RSV LRTIs in a very prematurely born cohort born at a median of 28 weeks of GA (Broughton et al. 2007). In this study, RSV LRTI results have not been reported separately, as the majority of the infants who had had a RSV LRTI also had another viral LRTI. The current infants who had suffered viral LRTIs were born at a median GA of 32 weeks and only six of them had been hospitalised. Hence, the results highlight that even mild viral LRTIs in moderately prematurely born infants are associated with reduced lung function at follow up.

No significant diffferences were found in FRC_{pleth} between the two groups. One previous study (Seidenberg et al. 1989) found differences in FRC_{pleth} between infants with and without viral LRTIs in infants, but only the results of the 14 infants with viral LRTIs were compared with six controls. Other studies (Stokes et al. 1981; Henry et al. 1983; Caswell et al. 1990) have

found an elevated FRC_{pleth} at follow up compared to the reference ranges but comparison was not made with a contemporaneous control group in any of these studies. In this study it is possible the infants in the viral LRTI group had smaller lungs that were relatively hyperinflated and thus there were no significant differences in FRC_{pleth} between the two groups, but there were no significant differences in the FRC_{He} or FRC_{MBW}.

Most studies demonstrating lung function abnormalities at follow up have reported only infants who were hospitalised because of their LRTI (Stokes et al. 1981; Henry et al. 1983; Hall et al. 1984; Cassimos et al. 2008; Sigurs et al. 2010). There have, however, been some studies of term born infants which have included non hospitalised infants (Tepper et al. 1992; McConnochie et al. 1985; Stein et al. 1999). In one study, infants who had mild bronchiolitis were demonstrated not to be at increased risk between the ages of 8 and 12 years for airway hyperreactivity or abnormalities in lung function (McConnochie et al. 1985). Similarly, in another study, although RSV LRTIs were associated with an increased risk of wheeze at age six, the risk decreased markedly with age and was no longer significant at age 13 (Stein et al. 1999). Reduced maximal flow at FRC ($V_{max}FRC$) and increased airway responsiveness to metacholine challenge, however, were demonstrated in 18 infants during infancy, nine of whom were not hospitalised, ten months after an episode of bronchiolitis (Tepper et al. 1992). In none of those studies were there attempts to identify respiratory

viruses. Those results (Tepper et al. 1992; McConnochie et al. 1985; Stein et al. 1999) may mean that viral LRTIs cause chronic morbidity only in early childhood. Further longitudinal studies are required to test that hypothesis, as it is possible that rhinovirus LRTIs may have longer term adverse effects. In the COAST study (Guilbert et al. 2011), infants at high risk of developing wheeze (at least one atopic parent) were followed up. Infants who wheezed with a rhinovirus infection in the first three years after birth were demonstrated to have reduced lung function as assessed by spirometry at five to eight years of age compared to those infants without a rhinovirus "wheezy" illness. In another study (Kotaniemi-Syrjänen et al. 2008), hospitalisation for "wheezy" rhinovirus infections in the first two years after birth was a risk factor for reduced lung function (increased bronchial responsiveness to exercise) at eight years of age. In the current study the rhinovirus results have not been analysed separately but, of note, the viral LRTI group had had rhinovirus LRTIs on nine occasions.

Some studies have shown a decline in lung function during infancy in prematurely born infants (Hofhuis et al. 2002; Hoo et al. 2002). In one (Hoo et al. 2002), there was a decline in small airway function as assessed by $V_{max}FRC$ over the first few months after birth in infants born between 32 and 35 weeks of GA who had no initial respiratory problems. In another, which included very prematurely born infants who had BPD, there was a decline in $V_{max}FRC$ between 6 and 12 months in those who had initially been

supported by conventional ventilation (Hofhuis et al. 2002). In this study, R_{rs}, C_{rs} and LCI results, improved between 36 weeks PMA and one year corrected age in both groups.

This study has a number of strengths and some limitations. Consecutive infants were recruited whose parents gave informed consent. The infants tended to be relatively "mature" prematurely born infants and hence, not surprisingly, only a small proportion was admitted to hospital because of the LRTI. Infants were followed prospectively and hence LRTIs were "captured" regardless of whether the infants required hospitalisation or the infants remained in the community. A wide variety of respiratory viruses was tested for, which highlighted that the majority of infants who had RSV LRTIs also had other viral LRTIs. Hence, it is not possible report whether there was any impact of RSV LRTIs alone on lung function at follow up. The multiple breath wash-in/out data at 36 weeks corrected age had a high co-efficient of variation (FRC_{MBW} 16% and LCI 13%). Comparisons of the results at 36 weeks postnatal age and one year corrected age, therefore, were not attempted.

In conclusion, these results suggest viral LRTIs, regardless of hospitalisation, adversely affect prematurely born infants' lung function at follow up. This may make them at greater risk of poorer outcomes with subsequent viral LRTIs.

Chapter 6: Pandemic influenza A (H1N1) virus 2009 in a prospectively followed cohort of prematurely born infants

6.1 Introduction

Pandemic influenza A (H1N1) virus was first reported in the United Kingdom in April 2009, with peaks of infection occurring in July and October 2009 (Health Protection Agency 2010b). Children less than five years of age and those with pre-existing lung disease were at the greatest risk for hospitalisation and mortality because of H1N1 (Jain et al. 2009; Sachedina & Donaldson 2010). Infants born prematurely have an increased risk of hospitalisation for lower respiratory tract infections (LRTIs) because of respiratory syncytial virus (RSV) in the first two years after birth than infants born at term (Boyce et al. 2000). The primary aim of this study, therefore, was to test the hypothesis that prematurely born infants would be more likely to be hospitalised with H1N1 or other viral LRTIs than term born infants.

Pandemic influenza A H1N1 immunisation became widely available in October 2009. The Department of Health and Joint Committee on Vaccination and Immunisation advised immunising children older than six months who were in high-risk groups, for example, those with a chronic lung disease such as bronchopulmonary dysplasia (BPD). A secondary aim of the study was to determine the rate of uptake of H1N1 immunisation in prematurely born infants and whether it was higher in those with BPD.

6.2 Methods

Prematurely born infants born at less than 36 weeks of gestational age (GA) were eligible for entry into the study if they were born between April and September in 2008 or 2009, that is, outside the RSV season. Consecutive infants, whose parents gave informed written consent, were recruited. After discharge from the neonatal/maternity unit infants were prospectively followed as described in section 2.1. Multiplex real-time reverse transcription polymerase chain reaction (PCR) was performed on the NPAs for nine virus types (influenza A and B, RSV A and B, human metapneumovirus, rhinoviruses, parainfluenza viruses 1–3) and real-time PCR was performed for adenoviruses as described in section 2.7. A separate multiplex real-time reverse transcriptase PCR that was in routine clinical use was used to test all samples over the pandemic period for influenza A (H1N1)v 2009. The multiplex tested for the haemaglutinin (H1) gene, a matrix gene and an MS2 phage internal control to test for inhibition of any samples. The primers and probes (all synthesised by Metabion, Martinsried, Germany) for the H1 and matrix genes and MS2 phage internal control are shown in Table 6.1.

Table 6.1: Sequences of the primers and probes used for the pandemic influenza A (H1N1)v 2009 PCR assays

Gene	Primer/Probe	Sequences
H1	Forward primer	5' TTA CCA GAT TTT GGC GAT CTA YT 3'
H1	Reverse primer	5' CCA GGG AGA CTA SCA RTA CCA 3'
H1	TaqMan Probe	5' 6 FAM-ACW GTC GCC AGT TC-MGBFQ
Matrix	Forward primer	5' GAC CRA TCC TGT CAC CTC TGA C 3'
Matrix	Reverse primer	5' AGG GCA TTY TGG ACA AAK CGT CTA 3'
Matrix	TaqMan Probe	Cy5-TGC AGT CCY CGC TCA CTG GGC ACG-BHQ 3
MS2	Forward primer	5' TGG CAC TAC CCC TCT CCG TAT TCA CG 3'
MS2	Reverse primer	5' GTA CGG GCG ACC CCA CGA TGA C 3'
MS2	TaqMan Probe	5' ROX- CAC ATC GAT AGA TCA AGG TGC CTA CAA GC-BHQ 2'

The primers were used at 10 pmoles and the probes at 5 pmoles for the H1 and matrix gene assays and the primers and probes were both used at 2 pmoles for the MS2 phage assay. The samples were tested using a Rotor-Gene 6000 real-time thermal cycler (QIAGEN, Crawley, UK) and the following conditions:

1. Hold at 50^{o}C for 30 minutes (reverse transcription)
2. Hold at 95^{o}C for 15 minutes (TaqMan polymerase activation and reverse transcriptase inactivation)
3. 40 cycles of:
    i. Step 1- 94^{o}C hold for 30 secs (denaturation)
    ii. Step 2- 60^{o}C hold for 30 secs, acquiring to cycle A (FAM, CY5, ROX)

Parents were contacted, and the GPs' and hospital notes were reviewed to investigate how many of the infants had received the pandemic influenza A H1N1 immunisation. Data collected on the prematurely born cohort from

April 2009, the start of the pandemic, until the end of March 2010, when only sporadic H1N1 cases were reported locally, were analysed for this study.

To ascertain the hospitalisation rate of term-born infants, the King's College Hospital (KCH) virology database was interrogated. All infants attending the emergency department had an NPA taken only if they required hospitalisation. All NPAs were tested as described above. The hospital records of all children with NPAs in which viruses were detected were then examined to determine which children were less than two years of age and born at term. Data concerning the number of children less than two years of age in the KCH catchment area were obtained from the Office for National Statistics.

6.3 Analysis

Differences were assessed for statistical significance using Fisher's exact test. Statistical analysis was performed using SPSS version 15 (SPSS Inc., Chicago, IL, USA).

6.4 Results

In all, 251 infants were eligible for entry into the study, but 74 parents refused consent and 27 did not complete the follow-up period. Those who

did or did not complete the study had similar gestational ages and birth weights. In total, 150 prematurely born infants with a median (range) GA of 34 (23–35) weeks and birth weight of 1880 (534–3446) g were prospectively followed; 16 had BPD (oxygen dependent beyond 28 days) (Jobe & Bancalari 2001). In all, 94 infants were born between April and September 2008 and 56 infants between April and September 2009. Only one (0.7%) infant developed a LRTI in which pandemic influenza A (H1N1)v 2009 RNA was detected in the NPA; he required hospitalisation. He was born at 24 weeks of GA, had BPD, and was two months post-term when he developed H1N1 infection. He was admitted to a paediatric ward for one night, but did not require any respiratory support. He had not received the pandemic influenza A H1N1 immunisation. Eight (5.3%) other infants were hospitalised with other known viral LRTIs (five RSV, one rhinovirus, one adenovirus, and one dual infection with adenovirus and rhinovirus), but tested negative for H1N1. Six infants had received palivizumab, including two infants who were hospitalised for a LRTI (one H1N1 and one RSV LRTI). During the study period, there were approximately 4513 children aged less than two years in the KCH catchment area. Three (0.07%) of the children born at term were admitted to KCH and had pandemic influenza A H1N1 virus infection. A further 29 (0.6%) term born children were admitted with other viral LRTIs (22 RSV, two human metapneumovirus, two parainfluenza type 1, one parainfluenza type 3, one influenza type B, and one dual infection of RSV and adenovirus). The rate of hospitalisation for pandemic influenza A H1N1

virus infection did not differ significantly between prematurely and term-born infants (0.7% versus 0.07%, p=0.12), but prematurely born infants had a higher rate of hospitalisation for other known viral LRTIs (5.3% versus 0.6%, p<0.0001), including RSV LRTIs (3.3% versus 0.5%, p<0.002). Fourteen (9.3%) infants in the prematurely born cohort received the H1N1 immunisation, including four (25%) of 16 infants with BPD. Significantly more prematurely born infants with BPD received the H1N1 immunisation compared with those without BPD (25% versus 7.5%, p=0.045).

6.5 Discussion

The results demonstrate that hospitalisation rates for pandemic influenza A H1N1 virus infections for prematurely born and term born infants did not differ significantly, but prematurely born infants had significantly higher rates of hospitalisation for other viral, including RSV, LRTIs. The study was particularly interested in the impact of RSV infection, and hence included only infants who had been exposed to an entire RSV season on discharge from the NICU. If infants born between September and April had been assessed, it is possible that higher rates of hospitalisation may have been found. Only one prematurely born infant was admitted because of H1N1 infection however, thus it is not possible to speculate on the influence of postnatal age in prematurely born infants with regard to the risk for H1N1 hospitalisation. The number of controls included in the study was adjusted to

take into account that the prematurely born infants were recruited only in six months of the year. A further limitation of this study was that some infants from other boroughs may have been admitted to KCH and infants born at KCH may have been admitted elsewhere. This is the first study in which pandemic influenza A H1N1 virus LRTI hospitalisations were prospectively collected in prematurely born infants. It was also found that pandemic influenza A H1N1 immunisation uptake was low in the prematurely born infants.

During the peak of the pandemic (July 2009), approximately 300 per 100,000 (0.3%) children aged less than one year attended their GP per week with an influenza-like-illness; a similar proportion of the children aged one to four years also attended their GP (Health Protection Agency 2010b). Miller et al (2010) reported 21.3% of children aged less than five years of age in London showed serological evidence of having had H1N1 infection. Certain of those children (Miller et al. 2010), however, may have been asymptomatic or had mild pandemic influenza A H1N1 virus infections not requiring hospitalisation, as samples were analyzed from children having blood tests for any reason. This study reports the hospitalisation rate for pandemic influenza A H1N1 virus LRTIs in term-born infants, and this was very similar to that reported for children less than five years of age (28 per 100,000; 0.028%) (Health Protection Agency 2010a).

Only 9.3% of the prematurely born cohort received the pandemic influenza A H1N1 immunisation. Although the proportion of infants with BPD receiving the immunization was higher (25%), these findings suggest that targeting of high-risk groups needs to be more effective.

In conclusion, pandemic influenza A H1N1 virus LRTI was not, but other viral LRTIs were, associated with a higher hospitalisation rate in prematurely born compared with term born infants. These data suggest that prematurity per se might not be a risk factor for increased hospitalisation with pandemic influenza A H1N1 virus infection.

Chapter 7: Rhinovirus infection and healthcare utilisation in prematurely born infants

7.1 Introduction

Respiratory syncytial virus (RSV) lower respiratory tract infections (LRTIs) are associated with increased chronic respiratory morbidity and healthcare utilisation in prematurely born infants who did (Greenough et al. 2009) or did not (Broughton et al. 2005; Broughton et al. 2007) develop bronchopulmonary dysplasia (BPD). Amongst those who had had BPD, healthcare utilisation and the related cost of care were increased up to seven years of age and lung function was lower than that of controls at ten years of age (Greenough et al. 2009). In term born children, rhinovirus (HRV) infection has also been associated with chronic respiratory morbidity (Lemanske et al. 2005; Jackson et al. 2008; Jartti & Korppi 2011; Guilbert et al. 2011; Midulla et al. 2012). There is some evidence that HRV infection may also have long term adverse effects on prematurely born infants, in that eight prematurely born infants with BPD who developed HRV LRTIs subsequently had a sustained worsening of their clinical status, requiring the addition of new therapies for prolonged periods of time (Chidekel et al. 1997). As a consequence, it was hypothesised that prematurely born infants with or without BPD who suffered a HRV LRTI would suffer increased healthcare utilisation in infancy and have greater health related cost of care. The aim of this study was to test that hypothesis. In addition, the study aimed to determine whether the magnitude of any increase in healthcare utilisation and the health related cost of care was similar to that associated with RSV LRTI.

7.2 Methods

Infants born less than 36 weeks of GA in 2008 or 2009 were eligible for entry into the study if they were born prior to the onset of the RSV season. The RSV season was defined as 1 October to 31 March, consistent with the UK experience. Following neonatal unit discharge, infants were followed prospectively until one year of corrected age as described in section 2.1. A researcher visited the home on every occasion that an infant had a LRTI and a nasopharyngeal aspirate (NPA) was obtained if the LRTI was confirmed by the researcher. NPAs were obtained on each occasion an infant was admitted with an LRTI. Real time PCR was performed on the NPAs for 13 viruses as described in section 2.7.

Follow up costs after neonatal discharge were calculated using the NHS reference costing scheme (2007-8) (Department of Health 2009) and the British National Formulary for Children (2008) as described in section 2.5. The respiratory costs were also reported and these were defined as costs related to a LRTI episode regardless of whether this was incurred in hospital or the community, e.g. a general practitioner (GP) attendance. Regarding healthcare utilisation, all visits to practice nurses or routine visits to health visitors, for example for immunisations, were not recorded as these were considered usual care for infants. To calculate the cost of care, the NHS reference costing scheme was used which gives national average costs for in- and out-patient hospital attendances and GP attendances. For

admissions, the number of days for each admission was multiplied by the national average cost for the diagnosis leading to admission.

Parents completed a respiratory diary card for one month when their infant was 11 months of corrected age and a respiratory health related questionnaire about their infant when the infant was one year of corrected age as described in section 2.6.

7.3 Analysis

The infants were divided into four groups:

(i) Infants who never had a symptomatic LRTI (no LRTI).

(ii) Infants who had at least one LRTI from which HRV was detected from the NPA (HRV LRTI)

(iii) Infants who had at least one LRTI from which RSV was detected from the NPA (RSV LRTI).

(iv) Infants who had LRTI(s) with HRV and RSV detected from NPA(s) (HRV/RSV LRTI).

The results of infants who had other viral LRTIs, but not a HRV or RSV LRTI or had a symptomatic LRTI, but no virus was detected, were excluded from the analysis.

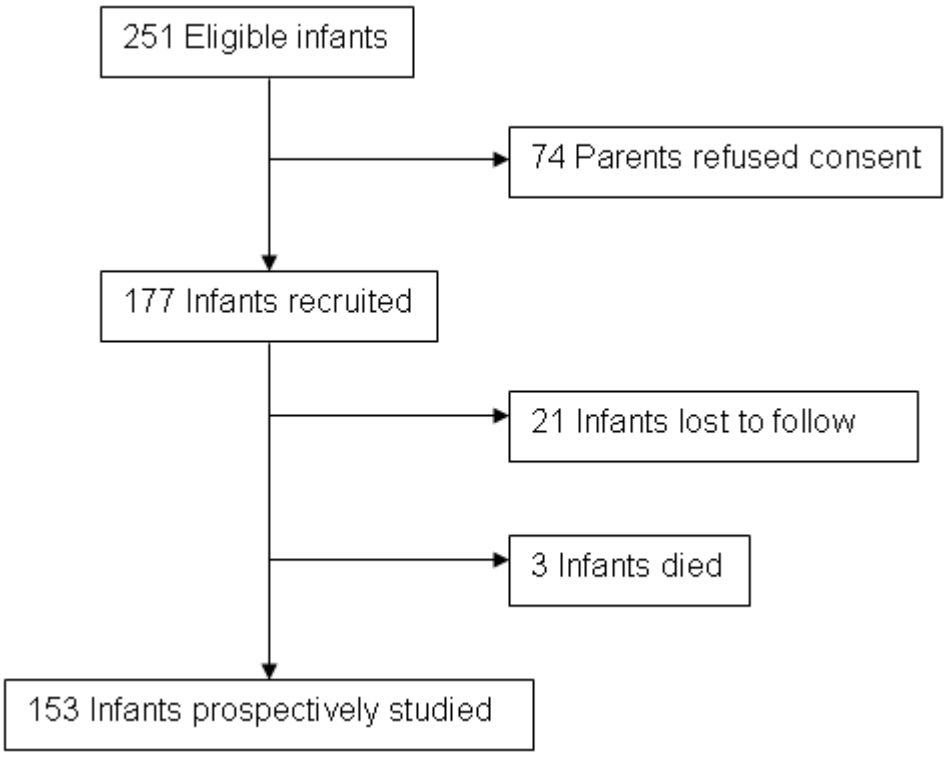
7.4 Statistical analysis

Baseline factors were compared across the four groups using the Kruskal-Wallis test with post-hoc tests adjusted for multiple comparisons using IBM SPSS Statistics (version 19, New York, USA). Further analysis was then carried out by Professor Janet Peacock and Jessica Lo using Stata (version 12.1 TX, USA). The cost data were summarised using means, rather than medians to preserve the total sum of costs (Thompson & Barber 2000). Cost data were fitted to a generalised linear model with a gamma distribution and identity link for the total cost data and a Poisson distribution with robust standard error and identity link for the respiratory cost data (Barber & Thompson 2004). To account for the differences in the demographics of the three groups, an adjusted analysis was performed with adjustment initially for birth weight, GA, antenatal steroid use and surfactant use, and then for those variables plus BPD. Principal components analysis was first used to reduce the birth weight, GA, antenatal steroid use and surfactant use data to two principal components that explained approximately 80% of the total variability in those factors. Those two components were then used as covariates in a further generalised linear model to obtain adjusted estimates. Adjustment was not possible for the respiratory data as none of the models would converge due to the dominance of nil costs in the no LRTI group.

7.5 Results

Two hundred and fifty one infants were eligible for inclusion into the study (Figure 7.1, Appendix 5).

Figure 7.1: Flow diagram of recruitment



The 153 (84 males) infants who completed the study had a median GA of 34 (range 23-35) weeks and a birth weight of 1890 (range 534-3610) g. Twenty infants developed HRV LRTIs, 17 developed RSV LRTIs, 12 developed both HRV/RSV LRTIs and 74 infants had no LRTI. Thirty infants had other viral or viral negative LRTIs and their results were excluded from the analysis. Of the 12 infants developing both HRV/RSV LRTIs, nine had HRV or RSV detected during two separate LRTIs and three infants had detection of HRV

and RSV during one LRTI. The HRV LRTI group had a median of two LRTIs, the RSV LRTI group a median of one LRTI and the HRV/RSV LRTI group a median of two LRTIs ($p<0.001$ across the three groups with post hoc analysis showing no significant difference between the HRV and the HRV/RSV LRTI groups, $p=0.95$, but significant differences between both those groups and the RSV LRTI group ($p<0.01$, $p<0.01$).

The significant differences in the demographics (Table 7.1) between the four groups were in GA (there was significant variability overall between the groups but there were no significant differences between pairs of groups), birth weight (the HRV LRTI group was significantly lighter at birth than the no LRTI group, $p<0.05$), antenatal steroid use (a significantly greater proportion of the mothers in the RSV group had received antenatal steroids than the no LRTI group, $p<0.05$), surfactant use (a greater proportion of the HRV/RSV LRTI group had received surfactant than either the RSV LRTI [$p<0.05$] or the no LRTI groups [$p<0.05$]), BPD (there was significant variability overall between the groups but there were no significant differences between pairs of groups) and palivizumab use (a significantly greater proportion of the HRV LRTI group had received palivizumab than the no LRTI group, $p<0.05$).

Nine infants (12%) of the no LRTI group had hospital admissions (all for non-respiratory causes), two (10%) of the HRV LRTI group had hospital admissions (one for a HRV LRTI and one for a non-respiratory cause), seven (41%) of the RSV LRTI group had hospital admissions (five for RSV

LRTI, one for another viral LRTI and one for a non-respiratory cause) and six (50%) of the HRV/RSV LRTI group had hospital admissions (two for a HRV/RSV LRTI, three for a RSV LRTI and one for a HRV LRTI).

The HRV LRTI group had more total ($p<0.05$) and respiratory related ($p<0.05$) out-patient attendances and general practitioner (GP) respiratory related attendances ($p<0.05$) than the no LRTI group and more respiratory related out-patient attendances than the RSV LRTI group ($p<0.05$). The RSV LRTI group had more total ($p<0.05$) and respiratory related ($p<0.05$) hospitalisations than the no LRTI group and more total ($p<0.05$) and respiratory related ($p<0.05$) hospitalisations than the HRV LRTI group. The RSV LRTI group had more total ($p<0.05$) and respiratory related ($p<0.05$) A and E attendances than the no LRTI group (Table 7.2). The HRV/RSV group had more total and respiratory related hospitalisations than both the no LRTI ($p<0.05$) and HRV LRTI ($p<0.05$) groups. The HRV/RSV LRTI group had more total ($p<0.05$) and respiratory related ($p<0.05$) A and E attendances than the no LRTI group (Table 7.2). The HRV/RSV LRTI group had more total out-patient attendances than the no LRTI group ($p<0.05$) (Table 7.2).

Analysis of the diary card data highlighted that the HRV/RSV LRTI group had more days of inhaler use than any of the other three groups ($p<0.05$, $p<0.05$ and $p<0.05$) (Table 7.3). Analysis of the respiratory health-related questionnaire data (Table 7.4) demonstrated that a greater proportion of the

HRV LRTI group wheezed than the no LRTI group ($p<0.001$). A greater proportion of the HRV/RSV LRTI group wheezed ($p<0.001$) and used bronchodilators ($p<0.01$) or preventers ($p<0.001$) than the no LRTI group (Table 7.4). A greater proportion of the HRV/RSV LRTI group used preventers compared to the RSV LRTI group ($p<0.001$) (Table 7.4).

There were significant differences overall across the four groups in the mean costs for out-patient attendances, GP respiratory attendances and medication (Table 7.5). The costs for out-patient attendances were greater in the HRV LRTI group compared to the no LRTI group ($p<0.05$) and compared to the RSV LRTI group ($p<0.05$). There were no significant differences between the four groups on post hoc analysis for the costs for GP respiratory attendances or medications.

Compared to the no LRTI group, the HRV/RSV LRTI group had the highest mean cost (difference = £7035), then the HRV LRTI group (difference = £1086), followed by the RSV LRTI group (difference = £678). These differences were reduced after adjusting for birth weight, GA, antenatal steroid and surfactant use, but overall the differences remained statistically significant. Further adjustment for BPD in addition, reduced the differences slightly more, but the overall differences in costs between groups remained significant ($p=0.045$) (Table 7.6). The health related cost of care of the HRV LRTI group was similar to that of the RSV LRTI group ($p=0.83$) (Table 7.6). The ordering of costs was the same for total respiratory costs and the

differences in mean costs were statistically significant (p=0.003) (Table 7.7). Adjustment for neonatal factors for total respiratory costs was not possible as models with possible confounders would not converge.

Table 7.1: Demographic data according to LRTI status

Data presented as median (range) or n (%).

	No LRTI	HRV LRTI	RSV LRTI	HRV/RSV LRTI	p*
n	74	20	17	12	
Gestational age (weeks)	34.1 (25.7-35.9)	32.9 (27.4-35.7)	32.6 (28.9-35.6)	32.1 (23.0-35.9)	0.044
Birth weight (g)	2070 (895-3610)	1558 (670-2512)	1756 (1080-2650)	1595 (610-2546)	0.002
Males	39 (53%)	9 (45%)	9 (53%)	5 (42%)	0.85
Antenatal smoking	11 (15%)	4 (20%)	2 (12%)	2 (17%)	0.91
Antenatal steroids	40 (54%)	16 (80%)	16 (94%)	8 (67%)	0.014
Surfactant	11 (15%)	7 (35%)	1 (6%)	6 (50%)	0.005
Duration of ventilation (days)	0 (0-82)	1 (0-103)	1 (0-17)	1.5 (0-81)	0.39
Bronchopulmonary dysplasia	4 (5%)	5 (25%)	0 (0%)	3 (25%)	0.008
Family history of atopy	52 (70%)	13 (65%)	8 (47%)	7 (58%)	0.32
Day Care	2 (3%)	0 (0%)	1 (6%)	1 (8%)	0.52
Number of siblings	1 (0-5)	1 (0-5)	1 (0-4)	1 (0-4)	0.91
Palivizumab	0 (0%)	3 (15%)	0 (0%)	1 (8%)	0.005

*The p values relate to the difference between the four groups, post hoc p values are given in the results section text.

Table 7.2: Healthcare utilisation to one year corrected age according to LRTI status

Data are presented as median [mean] (range).

	No LRTI	HRV LRTI	RSV LRTI	HRV/RSV LRTI	p*
n	74	20	17	12	
Admissions					
Total	0 [0.1] (0-2)	0 [0.1] (0-1)	0 [0.6] (0-3)	1 [1.8] (0-11)	0.001
Respiratory	0 [0] (0-0)	0 [0.1](0-1)	0 [0.4] (0-1)	0 [1.3] (0-11)	<0.001
A and E attendances					
Total	0 [0.7] (0-6)	1 [1.2] (0-5)	1 [1.6] (0-8)	1 [2.5] (0-14)	0.015
Respiratory	0 [0.1] (0-3)	0 [0.3] (0-4)	0 [0.7] (0-3)	1 [1.9] (0-12)	<0.001
Outpatient attendances					
Total	2 [3.3] (0-14)	5 [6.9] (0-18)	4 [4.0] (0-10)	5 [7.3] (1-15)	0.002
Respiratory	0 [0] (0-0)	0 [1.1] (0-9)	0 [0] (0-0)	0 [0.8] (0-8)	<0.001
General Practice attendances					
Total	5 [5.3](0-20)	6 [6.8](1-15)	5 [6.1](2-14)	9 [8.3](3-15)	0.069
Respiratory	0 [0.4] (0-2)	1 [1.4] (0-4)	1 [0.8](0-3)	1 [1.8](0-6)	0.002

* The p values relate to the difference between the four groups, post hoc p values are given in the results section text.

Table 7.3: Diary card data at one year corrected age according to LRTI status

Data presented as median [mean] (range). Not all parents completed the diary card.

	No LRTI	HRV LRTI	RSV LRTI	HRV/RSV LRTI	p*
n	49	16	11	9	
Days of cough	0 [3.3] (0-31)	0.5 [4.9] (0-18)	0 [6.5] (0-23)	3 [7.8] (0-30)	0.40
Days of wheeze	0 [0.7] (0-12)	0 [2.4] (0-27)	0 [0.1] (0-1)	0 [4.4] (0-30)	0.035
Days using antibiotics	0 [0] (0-2)	0 [0] (0-0)	0 [0.6] (0-7)	0 [1.1] (0-5)	0.042
Days using inhalers	0 [0] (0-0)	0 [1.9] (0-30)	0 [0] (0-0)	0 [4.3] (0-30)	<0.001

* The p values relate to the difference between the four groups, post hoc p values are given in the results section text.

Table 7.4: Respiratory health related questionnaire data according to LRTI status

Data presented as n (%) responding yes to the question. Not all parents completed the questionnaire

	No LRTI	HRV LRTI	RSV LRTI	HRV/RSV LRTI	p*
n	71	19	17	12	
Did your child cough in the first year?	66 (93%)	19 (100%)	17 (100%)	12 (100%)	0.47
Did your child wheeze in the first year?	11 (15%)	13 (68%)	8 (47%)	9 (75%)	<0.001
Did your child use any medication for a chest problem in the first year?	17 (24%)	10 (53%)	9 (19%)	7 (58%)	0.008
Bronchodilators (e.g. salbutamol)?	6 (8%)	6 (32%)	6 (35%)	6 (50%)	<0.001
Antibiotics?	13 (18%)	9 (47%)	7 (37%)	6 (50%)	0.01
Preventers (e.g. steroids, montelukast)?	0 (0%)	2 (11%)	0 (0%)	3 (25%)	0.001
Has your child ever been diagnosed with asthma by a doctor?	0 (0%)	0 (0%)	0 (0%)	1 (8%)	0.10

* The p values relate to the difference between the four groups, post hoc p values are given in the results section text.

Table 7.5: Costs of care (UK £) to one year corrected age according to LRTI status

Data are presented as mean [SD].

	No LRTI	HRV LRTI	RSV LRTI	HRV/RSV LRTI	Overall p value
n	74	20	17	12	
Admission					
Total cost	188 [654]	139[450]	665 [1006]	5771 [10168]	0.51
Respiratory cost	0 [0]	48 [214]	337 [627]	3470 [7740]	0.83
A and E					
Total cost	59 [100]	109 [148]	158 [228]	286 [509]	0.09
Respiratory cost	5 [33]	35 [116]	77 [113]	247 [486]	0.73
Out-patient					
Total cost	445 [418]	1402 [1689]	522 [321]	1227 [1317]	0.002
Respiratory cost	0 [0]	605 [1444]	0 [0]	362 [1210]	Not able to calculate[1]
General practice					
Total cost	287 [252]	439 [380]	313 [196]	731 [931]	0.05
Respiratory cost	16 [25]	55 [52]	32 [37]	75 [95]	0.02
Medication					
Total cost	109 [173]	672 [1218]	104 [150]	719 [1334]	0.04
Respiratory cost	2 [3]	484 [1195]	3 [5]	286 [963]	0.09
Overall					
Total cost	980 [958]	2067 [1794]	1658 [1299]	8015 [12072]	0.001
Respiratory cost	27 [78]	740 [1477]	446 [691]	4153 [9335]	0.003

[1] Generalised linear model did not converge and so the p value is unobtainable

Table 7.6: Unadjusted and adjusted mean total costs (UK £) according to LRTI status

	Unadjusted mean difference[1] (UK £)	95% CI for difference	Adjusted mean difference[2] (UK £)	95% CI for difference	Adjusted mean difference[3] (UK £)	95% CI for difference
No LRTI (n=74)	Reference group	Overall p value: p=0.001		Overall p value: p=0.040		Overall p value: p=0.045
HRV LRTI (n=20)	1086	54 to 2019	323	-441 to 1087	278	-448 to 1005
RSV LRTI (n=17)	678	-141 to 1497	151	-655 to 957	172	-631 to 975
HRV/RSV LRTI (n=12)	7035	2497 to 11573	5851	1789 to 9912	5769	1711 to 9828

[1] Difference in means between the reference group and each other group in turn

[2] Adjustment for birth weight, GA, antenatal steroid use and surfactant use

[3] Adjustment for all as in [2] plus BPD

Table 7.7: Unadjusted mean difference in overall respiratory costs (UK £) according to LRTI status

	Unadjusted mean difference[1] (UK £)	95% CI for difference
No LRTI (n=74)	Reference group	Overall p value: p=0.003
HRV LRTI (n=20)	713	79 to 1346
RSV LRTI (n=17)	418	98 to 738
HRV/RSV LRTI (n=12)	4125	-952 to 9203

[1] Difference in means between the reference group and each other group in turn. An adjusted model could not be fitted due to convergence problems

7.6 Discussion

This study has demonstrated that prematurely born infants developing HRV LRTIs had increased health related cost of care during infancy compared to infants who did not develop a LRTI. The results demonstrating the HRV LRTI group suffered chronic respiratory morbidity are in keeping with previous findings in eight prematurely born infants (Chidekel et al. 1997). In that series, however, all of the infants were born very prematurely, had had BPD and were hospitalised with the HRV LRTI (Chidekel et al. 1997). The 20 infants in the HRV group presently studied, however, were born at a higher GA, only 25% had had BPD and only one of the 20 infants had been hospitalised with the HRV LRTI.

The HRV/RSV group, as the RSV group, had significantly more admissions and A and E attendances than the no LRTI group. It has been previously reported that infants who develop a dual infection either with RSV and human metapneumovirus (Semple et al. 2005) or RSV and HRV (Richard et al. 2008) were more likely to develop a severe LRTI, as evidenced by requirement for a PICU admission. In this study, however, only three of the infants had HRV and RSV detected during the same LRTI. The higher admission rate of the HRV/RSV group may reflect a functional predisposition to severe viral LRTIs. It has previously been shown that very prematurely born infants who developed a RSV LRTI and subsequent chronic respiratory morbidity had significantly worse premorbid lung function (Broughton et al. 2006). In infants born at term, poorer premorbid lung function, that is a

higher resistance of the respiratory system in the first two months after birth, was associated with an increased risk of the occurrence and duration of HRV-associated wheeze during infancy (van der Zalm et al. 2011). It is thus possible that poorer premorbid lung function might explain the chronic respiratory morbidity of the HRV group. In infants born at term, HRV seems to preferentially affect the lower airways, causing bronchiolitis in atopic children prone to wheezing (Einarsson et al. 1996; Gern & Busse 1999; Gern et al. 2000). It has been suggested that the reduced interferon-gamma (IFN-γ) responses in infancy in children with atopy may partly explain why atopy is a risk factor for HRV-induced wheezing (Jartti & Korppi 2011). Interferon responses in early life are inversely associated with the severity of viral respiratory illnesses (Papadopoulos et al. 2002). In addition, infants with low ex-vivo IFN-γ responses in early life are more likely to have frequent viral respiratory illnesses, including those associated with wheezing (Copenhaver et al. 2004). In this study, skin prick testing was not performed on the infants as parents of prematurely born infants frequently refuse skin prick testing in a research setting, as do prematurely born children (Greenough et al. 2009). There were not, however, any significant differences between the groups in family history of atopy or the proportion of infants who had eczema. Whether atopy predisposes prematurely born infants to HRV LRTIs needs further investigation.

This study has a number of strengths and some limitations. A large cohort of prematurely born infants was prospectively followed from birth to one year of corrected age. It was possible to investigate symptomatic LRTIs, not only in

hospitalised infants, but also in those with LRTIs in the community. This is important, as it has previously been demonstrated that infants with RSV LRTI not requiring hospitalisation also suffer increased respiratory morbidity (Broughton et al. 2005). In addition, the NPAs were tested by real time multiplex PCR assays that had the advantage of high sensitivity to detect a wide range of respiratory viruses (Copenhaver et al. 2004; Kuypers et al. 2006). A limitation of the study, however, is that NPAs were only obtained when the infants were symptomatic and thus it is not possible to comment as to whether asymptomatic respiratory viral infections increase healthcare utilisation at follow up. HRV can be shed from the respiratory tract for several weeks after an acute infection (Peltola et al. 2013) and thus it is possible a sample testing positive for HRV was not cause of the acute infection LRTI at the time the sample was taken but was from a recent, previous infection. Both the HRV LRTI and the HRV/RSV LRTI groups had a median of two LRTIs, but the RSV LRTI group had a median of one LRTI which might have influenced differences in the cost of care. The HRV LRTI and RSV LRTI groups, however, had similar costs of care, but the type of healthcare utilisation which resulted in the increased cost of care compared to the no LRTI group differed between the two groups. Infants were recruited before the RSV season to ensure all infants were exposed to a whole RSV season. All infants, however, were followed for one year so seasonal variations between viruses would not affect the results. The groups differed significantly in respect to certain of their demographics; the no LRTI group were more mature at birth, of higher birth weight and less likely to have received antenatal steroids. The findings of increased health related costs of care

compared to the no LRTI group, however, remained statistically significant after adjusting for neonatal factors.

In conclusion, HRV LRTIs in prematurely born infants were associated with an increased health related cost of care during infancy. The increased health related cost of care in the HRV LRTI group was due to more out-patient and respiratory related general practitioner attendances and a greater proportion of the group wheezed at follow up whereas in the RSV LRTI group it was due to more hospital admissions. Those data suggest the HRV LRTI group suffered greater chronic respiratory morbidity than the RSV LRTI group.

Chapter 8: Respiratory outcome of prematurely born infants following human rhinovirus A and C infections

8.1 Introduction

Human rhinoviruses (HRVs) are a common cause of lower respiratory tract infections (LRTIs) in both term (van der Zalm et al. 2011) and prematurely (Miller et al. 2012) born infants. HRVs had been classified into two genetic groups (HRV-A and HRV-B) comprising over 100 serotypes, with HRV-A being more prevalent than HRV-B. A further HRV group (HRV-C) has subsequently been identified by genomic nucleotide sequencing (Lee et al. 2007). HRV-C group viruses have been reported to have a similar prevalence to HRV-A in hospitalised (Fry et al. 2011; Iwane et al. 2011), ambulatory (Arden et al. 2010) and asymptomatic patients (Fry et al. 2011; Iwane et al. 2011). There are, however, conflicting data as to whether HRV-A or HRV-C infections causes more severe acute disease (Miller et al. 2009; Jin et al. 2009; Xiang et al. 2010). HRV LRTIs have also been associated with chronic respiratory morbidity in infants born at term or prematurely (Chidekel et al. 1997; Jackson et al. 2008; Guilbert et al. 2011). The aim of this study was to assess whether HRV group A or C viruses were associated with chronic respiratory morbidity and increased healthcare utilisation in prematurely born infants.

8.2 Methods

Infants born less than 36 weeks of gestation were eligible for entry into the study if they were born prior to the onset of the RSV season in 2008 or 2009. The RSV season was defined as from October 1st to March 31st as per UK

experience (Clark 2000). After neonatal/maternity unit discharge infants were followed until one year corrected age as described in section 2.1. Real time polymerase chain reaction (PCR) had been performed on the NPAs for 13 viruses as described in section 2.7. For the purposes of this study, the nucleic acid from the stored clinical specimens was subtyped for HRV A, B and C by Dr Ina Lauinger and Dr William Tong. RNA extraction was carried out using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis and PCR amplification of the VP4/VP2 region for typing was carried out as previously described (Lauinger et al. 2012). PCR products were cleaned with microClean (Microzone) before cycle sequencing with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on the automated sequencer 3130xl Genetic Analyzer (Applied Biosystems). Sequence analysis was carried out using the software BioEdit (version 7.0.9) and MEGA5 (version 5.03). Sequencing was performed in one direction using the forward primer. More than 10% divergence in VP4/VP2 is required to identify a different HRV type (Simmonds et al. 2010; McIntyre et al. 2013); this is well above the contribution of any potential polymerase error.

Parents completed a diary card for one month when their infant was 11 months corrected age and a respiratory health related questionnaire about their infant when the infant was one year corrected age as described in section 2.6. Follow up costs after neonatal discharge were calculated using the NHS reference costing scheme (2007-8) (Department of Health 2009) and the British National Formulary for Children (2008) as described in section

2.5. The respiratory costs were also reported and these were defined as costs related to a LRTI episode regardless of whether this was incurred in hospital or the community, e.g. a general practitioner (GP) attendance.

In addition, lung function (FRC_{He}, FRC_{MBW}, LCI, C_{rs}, R_{rs}, FRC_{pleth}, R_{aw} and $FRC_{He:pleth}$ ratio) was carried out at one year corrected age as described in section 2.4.

8.3 Analysis

Infants who had LRTIs for which no virus was detected were excluded from the analysis. If an infant had a LRTI with a virus other than HRV-A or HRV-C, but had not had an HRV-A or HRV-C LRTI they also were excluded from the analysis.

The remaining infants were divided into three groups:

(i) Infants who never had a symptomatic LRTI - no LRTI group (n=74)

(ii) Infants who had a LRTI with HRV-A detected from the NPA – HRV-A LRTI group (n=9)

(iii) Infants who had a LRTI with HRV-C detected from the NPA – HRV-C LRTI group (n=13)

Continuous data were tested for normality using the Shapiro-Wilk test and found not to be normally distributed. Differences, therefore, were assessed for statistical significance using Kruskal-Wallis test with a post hoc ANOVA

test. Categorical data were analysed using the Fisher's exact test. Statistical analysis was performed using IBM SPSS Statistics (version 19, New York, USA).

8.4 Results

One hundred and fifty three infants with a median gestational age (GA) of 34 (range 23-35) weeks and a birth weight of 1890 (range 534-3610) g were prospectively followed. Thirty two infants developed 40 HRV LRTIs (Figure 8.1, Appendix 5). Of the 40 HRV LRTIs, there were 29 samples from 24 infants with sufficient nucleic acid for subtyping (Table 8.1). There were no significant differences in the demographics of the infants who did and did not have subtyping of their HRV LRTIs. Nine infants had a HRV-A LRTI (one infant had two different HRV-A serotype LRTIs [serotypes 80 and 56], the LRTIs were three months apart), one infant had a HRV-B LRTI (serotype 42) and 13 infants had a HRV-C LRTI (one infant had two different HRV-C serotype LRTIs [serotypes C40 and C12], the LRTIs were three months apart). One infant had a HRV LRTI which was not possible to subtype and in three samples subtyping failed. Six of the infants in the HRV-A group and three of the infants in the HRV-C group also had RSV LRTIs (either as dual infections or as a separate LRTI). Other viral LRTIs in the HRV-A group were RSV A (n=5), RSV B (n=3), adenovirus (n=5), human metapneumovirus (n=1), influenza B (n=2), parainfluenza 1 (n=1), parainfluenza 3 (n=1), enterovirus (n=3), parechovirus (n=2) and for the HRV-C group were RSV A (n=2), RSV B (n=1), adenovirus (n=4), human metapneumovirus (n=2),

influenza A (n=1), influenza B (n=1), parainfluenza 1 (n=2), parainfluenza 3 (n=2), enterovirus (n=7), parechovirus (n=1) and human bocavirus (n=2).

Figure 8.1: Flow diagram of HRV subtype testing

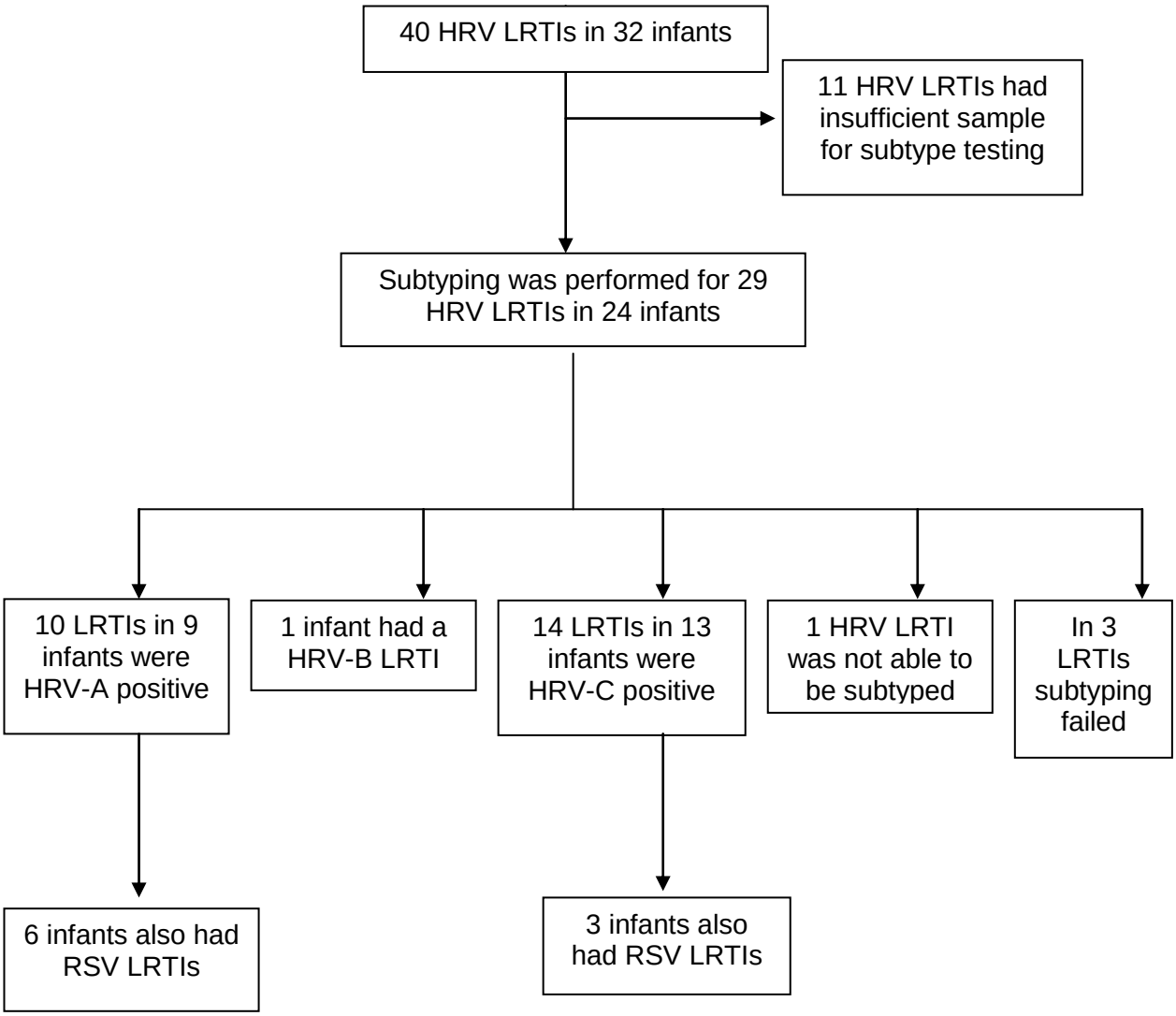


Table 8.1: HRV-A and HRV-C serotypes and the number of LRTIs in which a particular serotype was found

HRV-A serotype	Number of LRTIs	HRV-C serotype	Number of LRTIs
12	1	C2	1
16	1	C3	2
24	1	C5	1
56	1	C12	1
58	1	C14	1
61	1	C25	1
78	1	C36	1
80	2	C39	1
101-v1	1	C40	2
		C pat18	1
		C pat19	1
		C pat21	1

There were no statistically significant differences in the demographic data between the three groups, although both HRV groups tended to be lighter at birth (p=0.056) and a greater proportion of them tended to have received surfactant (p=0.050) or palivizumab (p=0.051) than the no LRTI group (Table 8.2). Three (33%) of the HRV-A LRTI group had hospital admissions (one for a RSV LRTI, one for a HRV-A/enterovirus dual LRTI and one had multiple admissions for viral LRTIs including RSV and HRV-A). Three (23%) of the HRV-C LRTI group had hospital admissions (one for an adenovirus/human bocavirus dual LRTI, one for a RSV/human metapneumovirus/HRV [unsubtyped] triple LRTI and one for a non-respiratory cause). Nine (12%) infants of the no LRTI group had hospital admissions, all for non-respiratory causes.

Analysis of the diary card data demonstrated that the HRV-A LRTI group had significantly more days of antibiotic (p=0.021) and inhaler (p<0.0005) use than the no LRTI group (Table 8.3). Exclusion of infants who had had a RSV LRTI from the analysis, however, demonstrated no significant differences between the HRV-A LRTI group and the no LRTI group (Table 8.4), but there were only three infants who had a HRV-A LRTI who did not also have a RSV LRTI. Compared to the no LRTI group, infants in the HRV-C LRTI group tended to have more days of cough (p=0.06) and wheeze (p=0.06) (Table 8.4). Exclusion of the infants who had a RSV LRTI from the analyses revealed that the HRV-C LRTI group had significantly more days of wheeze (p=0.01) and more days of inhaler use (p=0.024) than the no LRTI group (Table 8.4).

Analysis of the respiratory health related questionnaire demonstrated that compared to the no LRTI group, infants in the HRV-A LRTI group were more likely to wheeze ($p<0.0005$), use respiratory medications ($p<0.0005$), bronchodilators ($p=0.027$), antibiotics ($p=0.005$) or a preventer ($p=0.017$) (Table 8.3). Exclusion of infants who had had a RSV LRTI from the analysis, however, demonstrated no significant differences between the HRV-A LRTI group and the no LRTI group (Table 8.4). Compared to the no LRTI group, infants in the HRV-C LRTI group were more likely to wheeze ($p<0.0005$), use respiratory medications ($p<0.0005$), bronchodilators ($p<0.0005$), antibiotics ($p=0.008$) or a preventer ($p=0.002$) (Table 8.3). Exclusion of infants who had had a RSV LRTI from the analyses revealed that, compared to the no LRTI group, the ten remaining infants in the HRV-C LRTI group were more likely to wheeze ($p<0.0005$), use respiratory medications ($p=0.001$), bronchodilators ($p<0.0005$), antibiotics ($p=0.002$) or a preventer ($p<0.0005$) (Table 8.4).

The respiratory cost of care was significantly greater for both the HRV-A ($p=0.003$) and the HRV-C ($p<0.0005$) LRTI groups compared to the no LRTI group. When the infants who had had a RSV LRTI were excluded from the analysis, however, the respiratory cost of care compared to the no LRTI group was only significantly greater for the HRV-C LRTI group ($p<0.0005$) (Table 8.5). The total cost of care compared to the no LRTI group was significantly greater only for the HRV-C LRTI group ($p=0.017$), but the difference was not statistically significant when infants who had a RSV LRTI were excluded from the analysis (Table 8.5).

The only significant difference in the lung function between the groups was in R_{aw} where the HRV-C group had significantly higher R_{aw} than the no LRTI group (p=0.046) but the difference was not statistically significant when infants who had a RSV LRTI were excluded from the analysis (p=0.16) (data not shown).

Table 8.2: Demographic data according to viral LRTI status

Data are expressed as median (range) or n (%).

	HRV-A LRTI	HRV-C LRTI	No LRTI	p
n	9	13	74	
Gestational age (weeks)	34.4 (23.0-35.7)	32.3 (24.9-35.9)	34.1 (25.7-35.9)	0.39
Birth weight (g)	1826 (610-2546)	1729 (670-2512)	2070 (895-3610)	0.056
Males	4 (44%)	5 (39%)	39 (53%)	0.68
Antenatal smoking	1 (11%)	2 (15%)	11 (15%)	>0.99
Antenatal steroids	7 (78%)	10 (77%)	40 (54%)	0.28
Surfactant	4 (44%)	4 (31%)	11 (15%)	0.050
Duration of ventilation (days)	2 (0-81)	0 (0-103)	0 (0-82)	0.59
Bronchopulmonary dysplasia	2 (22%)	2 (15%)	4 (5%)	0.09
Family history of atopy	4 (44%)	8 (62%)	52 (70%)	0.27
Number of siblings	1 (0-4)	1 (0-5)	1 (0-5)	0.51
Breastfed	6 (67%)	10 (77%)	62 (84%)	0.39
Palivizumab	1 (11%)	1 (8%)	0 (0%)	0.051

Table 8.3: Diary card and respiratory health related questionnaire data by HRV subtype

Data presented as median (range) or n (%) responding yes to the question.

	HRV-A LRTI	HRV-C LRTI	No LRTI	p**
		Diary card data*		
n	9	8	49	
Days of cough	3 (0-30)	5 (0-17)	0 (0-31)	0.032
Days of wheeze	0 (0-30)	0.5 (0-7)	0 (0-12)	0.009
Days using antibiotics	0 (0-5)	0 (0-0)	0 (0-2)	0.021
Days using inhalers	0 (0-30)	0 (0-30)	0 (0-0)	0.001
Days of acute GP/hospital attendance	0 (0-5)	0 (0-3)	0 (0-1)	0.050
		Respiratory health related questionnaire data *		
n	9	13	71	
Did your child cough in the first year?	9 (100%)	13 (100%)	66 (93%)	>0.99
Did your child wheeze in the first year?	7 (78%)	10 (77%)	11 (15%)	<0.0005
Did your child use any medication for a chest problem in the first year?	7 (78%)	9 (69%)	17 (24%)	<0.0005
Bronchodilators (e.g. salbutamol)?	4 (44%)	7 (54%)	6 (8%)	<0.0005
Antibiotics?	6 (67%)	8 (62%)	13 (18%)	<0.0005
Preventers (e.g. steroids, montelukast)?	2 (22%)	3 (23%)	0 (0%)	0.001
Has your child ever been diagnosed with asthma by a doctor?	1 (11%)	0 (0%)	0 (0%)	0.097

*Diary card and respiratory health-related questionnaire data were not available for all infants

** The p values relate to the difference between the three groups, post hoc p values are given in the results section text.

Table 8.4: Diary card and respiratory health related data by HRV subtype excluding infants who had had a RSV LRTI

Data presented as median (range) or n (%) responding yes to the question.

	HRV-A LRTI	HRV-C LRTI	No LRTI	p**
		Diary card data*		
n	3	7	49	
Days of cough	1 (0-3)	8 (0-17)	0 (0-31)	0.16
Days of wheeze	0 (0)	1 (0-7)	0 (0-12)	0.01
Days using antibiotics	0 (0)	0 (0-0)	0 (0-2)	0.90
Days using inhalers	0 (0)	0 (0-30)	0 (0-0)	0.024
Days of acute GP/hospital attendance	0 (0)	0 (0-3)	0 (0-1)	0.34
		Respiratory health related questionnaire data*		
n	3	10	71	
Did your child cough in the first year?	3 (100%)	10 (100%)	66 (93%)	>0.99
Did your child wheeze in the first year?	2 (67%)	8 (80%)	11 (15%)	<0.0005
Did your child use any medication for a chest problem in the first year?	2 (67%)	8 (80%)	17 (24%)	<0.0005
Bronchodilators (e.g. salbutamol)?	0 (0%)	6 (60%)	6 (8%)	0.001
Antibiotics?	62(67%)	7 (70%)	13 (18%)	0.001
Preventers (e.g. steroids, montelukast)?	0 (0%)	2 (22%)	0 (0%)	0.022
Has your child ever been diagnosed with asthma by a doctor?	0 (0%)	0 (0%)	0 (0%)	>0.99

*Diary card and respiratory health related questionnaire data were not available for all infants

** The p values relate to the difference between the three groups, post hoc p values are given in the results section text.

Table 8.5: Health related cost of care (UK £) by HRV subtype

Data are presented as median [mean] (range).

	HRV-A LRTI	HRV-C LRTI	No LRTI	p*
Respiratory costs	110 [3951] (0-31527)	74 [1465] (0-12685)	0 [27] (0-599)	<0.0005
Total costs	817 [5039] (529-33176)	1834 [3032] (494-14242)	720 [980] (0-4253)	0.008
Results excluding infants who had had a RSV LRTI				
Respiratory costs	64 [58] (0-110)	78 [636] (36-3357)	0 [27] (0-599)	<0.0005
Total costs	817 [871] (529-1265)	1615 [2115] (494-5494)	720 [980] (0-4253)	0.052

* The p values relate to the difference between the three groups, post hoc p values are given in the results section text.

Table 8.6: Lung function results at one year corrected age by HRV subtype

Data are presented as median (range).

	HRV-A LRTI	HRV-C LRTI	No LRTI	p*
N**	4	8	38	
Corrected age (months)	13 (12.8-13.5)	13.5 (10.0-17.3)	13 (10-16)	0.42
Weight (kg)	10.0 (8.2-10.9)	9.5 (7.8-10.9)	9.7 (7.4-12.2)	0.76
Length (cm)	79.4 (72.0-81.5)	78.6 (71.3-82.6)	77.9 (71.8-83.1)	0.95
FRC_{He} (mL/kg)	25 (22-26)	25 (20-32)	25 (17-32)	0.99
FRC_{MBW} (mL/Kg)	22 (18-28)	21 (17-26)	22 (13-32)	0.91
LCI	7.3 (7.1-7.3)	7.2 (6.4-7.8)	7.2 (6.2-8.4)	0.98
C_{rs} (mL/cmH_2O/kg)	1.5 (1.4-1.9)	2.0 (1.2-2.6)	1.7 (1.0-4.0)	0.82
R_{rs} (cmH_2O/L/s)	45 (42-48)	42 (38-67)	42 (31-67)	0.24
FRC_{pleth} (mL/kg)	23 (21-30)	26 (21-39)	25 (19-36)	0.79
R_{aw} (cmH_2O/L/s)	20 (19-30)	22 (14-33)	16 (11-33)	0.026
$FRC_{He:pleth}$ ratio	0.84 (0.81-0.97)	0.89 (0.81-0.99)	0.89 (0.74-0.99)	0.86

**The p values relate to the difference between the three groups, post hoc p values are given in the results section text

** Not all infants had lung function tested.

8.5 Discussion

It has been demonstrated that infants who had a HRV-C LRTI compared to infants who did not have a LRTI were significantly more likely to wheeze and use respiratory medications and had a greater respiratory cost of care. Initial analysis demonstrated HRV-A LRTIs were associated apparently with similar effects, but the significant differences compared to the no LRTI group disappeared once the infants who also had RSV LRTIs were excluded from the analysis, but there were only three infants who had an HRV-A LRTI and not an RSV LRTI. The significant differences between the HRV-C and no LRTI groups remained after excluding infants who had had a RSV LRTI. Indeed, exclusion of the infants who also had had a RSV LRTI revealed HRV-C LRTIs were associated with significantly more days of wheeze and use of an inhaler. Initial analysis demonstrated HRV-C LRTIs were associated with reduced lung function (higher R_{aw}) at follow up, however, this difference disappeared after excluding the infants who also had an RSV LRTI. These results, thus suggest HRV-C is associated with increased chronic respiratory morbidity during infancy in prematurely born infants.

Certain studies (Miller et al. 2009; Arden et al. 2010), but not all have reported that HRV-C compared to HRV-A results in more severe initial illness. In this study, three infants in both the HRV-A and HRV-C groups were admitted to hospital, but only one infant was admitted solely for a HRV-A LRTI and none for a HRV-C LRTI, thus it is not possible to comment in this prematurely born population whether HRV-A or HRV-C is associated with

more severe acute disease. During the same time period, only one infant had a HRV-B LRTI, suggesting HRV-B infections are less common than HRV-A or HRV-C infections in prematurely born infants, as has been found in infants and children hospitalised with LRTIs and controls without respiratory symptoms (Fry et al. 2011; Iwane et al. 2011).

This study has a number of strengths and some limitations. Respiratory morbidity was assessed using a variety of techniques; telephone calls, questionnaires and diary cards. Certain similar questions were used during the follow up telephone calls and on the questionnaire. The questionnaire gave information about the infant's health throughout infancy, the diary card more detailed information for one month. Strengths include that NPAs were collected whenever the infants had an LRTI, regardless of whether this was in hospital or in the community. In addition, NPAs were tested for a wide range of respiratory viruses and, hence, it was possible to demonstrate that the apparent association of HRV-A LRTIs with chronic respiratory morbidity may be explained by the infants having also had RSV LRTIs. A limitation is that the nucleic acid was subtyped only after other investigations had been undertaken and this meant there was insufficient nucleic acid for subtyping in eight of the 32 infants who had had HRV LRTIs. The infants with and without subtyping of their HRV LRTIs, however, did not differ in their demographics. It is therefore likely the results are generalisable. The infants with HRV-A and HRV-C infections had a variety of other viral LRTIs which may have increased their respiratory morbidity. Analysis excluding all other respiratory viral LRTIs, except RSV LRTI, was not undertaken as there is very little

information on the long term outcomes of prematurely born infants following those infections. After excluding infants who also had an RSV LRTI, which is known to increase chronic respiratory morbidity, it has been highlighted that HRV-C LRTIs were associated with an adverse outcome.

In conclusion, it has been demonstrated that HRV-C LRTIs were associated with chronic respiratory morbidity during infancy in prematurely born infants. Whether there is a similar adverse outcome in infants born at term merits testing.

Chapter 9: Viral lower respiratory tract infections and preterm infants' healthcare utilisation

9.1 Introduction

A prospective study has demonstrated increased healthcare utilisation following RSV LRTI in the first year after birth (Broughton et al. 2005). Retrospective studies have demonstrated that infants born moderately prematurely had increased healthcare utilisation in the first two years after birth (Shefali-Patel et al. 2012) and those who had BPD may have a similar adverse outcome even at school age (Greenough et al. 2009). Groothuis et al demonstrated that certain prematurely born infants with BPD who were in a home oxygen programme also required hospitalisation for RSV LRTI in the second year after birth and their hospitalisations were prolonged and complicated (Groothuis et al. 1988). Those data (Groothuis et al. 1988; Greenough et al. 2009) then suggest that the cost of care in the second year may also be increased following RSV LRTIs. The aim of this study was to test that hypothesis, as such data would inform the cost-benefit of prophylaxis in the second year. It has also been demonstrated that rhinovirus (HRV) LRTIs increase healthcare utilisation in prematurely born infants after neonatal unit discharge (Miller et al. 2012). A subsidiary aim then of this study was to determine whether other viral LRTIs, in particular HRV, might also be associated with an increased health related cost of care in the second year after birth. This study, therefore, determined healthcare utilisation and the health related cost of care in the first two years after birth in infants who developed a RSV LRTI, another viral LRTI or no LRTI in infancy and compared the costs in year one and year two.

9.2 Methods

Infants born less than 36 weeks of gestation were eligible for entry into the study if they were born prior to the onset of the RSV season in 2008. The RSV season was defined as from October 1st to March 31st as per UK experience (Clark 2000). After neonatal/maternity unit discharge infants were followed until two years corrected age as described in section 2.1. Real time polymerase chain reaction (PCR) was performed on the NPAs for 13 viruses as described in section 2.7. Parents completed a diary card for one month when their infant was 23 months corrected age and a respiratory health related questionnaire when their infant was two years of corrected age, regarding whether their infant had cough and/or wheeze, use of respiratory related medications (e.g. inhalers, oral steroids, antibiotics) in their second year after birth and whether their infant had been diagnosed by a doctor as having asthma as described in section 2.6.

Follow up costs after neonatal discharge were calculated using the NHS reference costing scheme (2007-8) (Department of Health 2009) and the British National Formulary for Children (2008) as described in section 2.5. The respiratory costs were also reported and these were defined as costs related to a LRTI episode regardless of whether this was incurred in hospital or the community, e.g. a general practitioner (GP) attendance.

During the first year after birth, infants with bronchopulmonary dysplasia (BPD) who had required supplementary oxygen until at least one week before NICU discharge and were being discharged during the RSV season

were given palivizumab. No infant received palivizumab in the second year after birth.

9.3 Analysis

The infants were divided into three groups:

(i) Infants who did not have a symptomatic LRTI in the first two years (no LRTI group).

(ii) Infants who had at least one RSV LRTI in the first year after birth. In the second year they could have had no LRTI, a RSV or another viral LRTI (RSV group).

(iii) Infants who had at least one LRTI with viruses other than RSV detected from the NPA in the first year and no RSV LRTI in the second year (other viral group).

A sub-analysis was undertaken of the infants who had had a HRV LRTI.

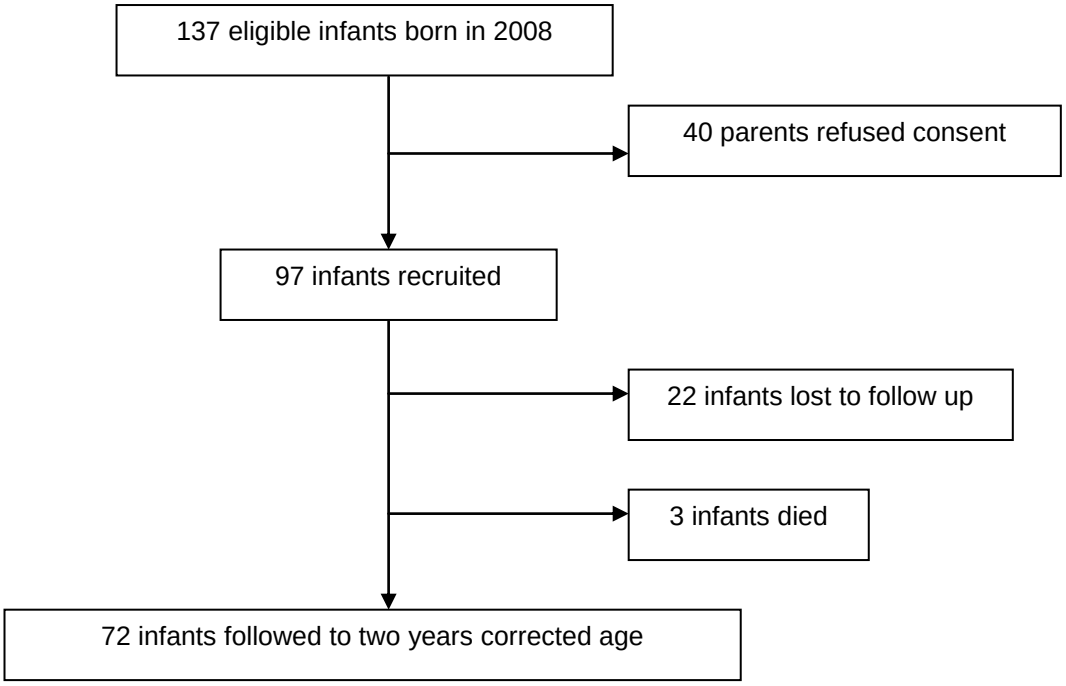
Baseline factors were compared across the three groups using the Kruskal-Wallis test with post-hoc tests adjusted for multiple comparisons. The cost data were summarised using means, rather than medians to preserve the total sum of costs (Thompson & Barber 2000). An attempt was made to analyse the cost data using the approach advocated by Barber and Thompson (Barber & Thompson 2004) by Professor Janet Peacock using Stata (version 12.1 TX, USA), however, due to a relatively small number of patients and the data being very skewed, a generalised linear model would not converge. The cost data, therefore, were analysed using the Kruskal-

Wallis test with post-hoc tests adjusted for multiple comparisons. The number need to treat (NNT) was calculated assuming a 50% reduction in the hospitalisation rate if RSV prophylaxis had been given (1998).

9.4 Results

One hundred and thirty seven infants were eligible for inclusion into this part of the cohort study. Ninety seven infants were recruited, three infants died before discharge and 22 were lost to follow up (Figure 9.1, Appendix 5).

Figure 9.1: Flow diagram of recruitment



Seventy two infants completed the two year follow up study, but certain infants were excluded from the analysis: those who had only viral negative LRTIs in the first year (n=6), infants who had no LRTI in the first year, but a viral negative LRTI (n=1), another viral LRTI (n=1) or a RSV LRTI (n=3) in

the second year after birth and infants who had other viral LRTIs in the first year and a RSV LRTI in the second year after birth (n=2). Thirteen of the infants developed RSV LRTIs (RSV group). Twenty-one of the infants developed other viral LRTIs (viruses detected were: HRV n=16; enterovirus n=7; parainfluenza type 3 n=6; adenovirus n=5; human metapneumovirus n=4; parechovirus n=2; human bocavirus n=2; parainfluenza type 1 n=2; influenza A n=2; influenza B n=1. Eight infants had dual infections and one a triple infection. Twenty five infants had no LRTIs in either year (no LRTI group, n=25). The median GA of the infants included in this study was 34 (23-35) weeks. There were no significant differences in the demographic data of the three groups (Table 9.1).

There were no significant differences in the number of LRTIs in the first year (RSV group, median 2 [range 1-11] versus other viral LRTI group, median 2 [range 1-4], $p>0.99$), but the RSV group had more LRTIs in the second year (median 1 [range 0-7]) than the other viral LRTI group (median 0 [range 0-2]) ($p=0.017$).

In the first year, the RSV group had more total ($p<0.0005$, $p<0.0005$ respectively) and respiratory related ($p<0.0005$, $p=0.003$ respectively) admissions and respiratory related A and E attendances ($p=0.001$, $p<0.0005$ respectively) than the other viral group and the no LRTI group (Table 9.2). Three infants in the RSV group also developed RSV LRTIs in the second year, two were admitted. In the second year, the RSV group had more respiratory related hospital admissions ($p=0.01$, $p=0.007$ respectively) and A and E respiratory attendances ($p=0.002$, $p=0.001$ respectively) than the other

viral group and than the no LRTI group (Table 9.2). Only the RSV LRTI group had respiratory admissions in either year: year one (n=6) and year two (n=2). There were no significant differences in healthcare utilisation in the second year between the other viral group and the no LRTI group and between the infants with HRV LRTIs and the no LRTI group (data not shown).

In the first year, the RSV group had higher costs than the other viral group for total ($p<0.0005$) and respiratory ($p<0.0005$) admission costs and A and E respiratory costs ($p=0.001$) and higher costs than the no LRTI group for total ($p<0.0005$) and respiratory ($p=0.003$) admissions, A and E total ($p=0.029$) and respiratory ($p<0.0005$) costs and overall total ($p=0.01$) and respiratory ($p=0.041$) costs (Table 9.3). In the second year, the RSV group had significantly higher costs than the other viral group for respiratory admissions ($p=0.01$), A and E respiratory costs ($p=0.002$) and respiratory medications ($p=0.042$) and the no LRTI group for respiratory admissions ($p=0.007$), A and E respiratory costs ($p=0.001$), respiratory medications ($p=0.004$) and overall respiratory costs ($p=0.012$) (Table 9.3). The three infants who had RSV LRTIs in the second year had significantly higher overall respiratory costs that year than those in the RSV group who did not have RSV LRTIs in the second year (mean [SD] cost £4788 [6556] versus £96 [109], $p=0.011$). There were no significant differences in cost of care between the other viral group and the no LRTI group or between those infants who had a HRV LRTI and the no LRTI group (data not shown). Two of the 59 infants were admitted for a RSV infection in the second year after birth, that is, a RSV

admission rate of 3.4%. Assuming prophylaxis would reduce the hospitalisation rate by 50%, the number needed to treat (NNT) was 59.

All three groups had higher overall total costs in year one compared to year two (RSV p=0.017; other viral p=0.001, no LRTI p=0.004) (Table 9.3). There were no significant differences in overall respiratory costs in any of the groups between the first and second years (RSV p=0.57; other viral p=0.61; no LRTI p=0.40) (Table 9.3).

A greater proportion of the RSV group had wheeze (p=0.014) and a doctor diagnosis of asthma (p=0.041) in the second year than the no LRTI group (Table 9.4). There were no significant differences between the RSV and other viral LRTI groups. Too few parents completed the diary card at two years of corrected age to enable any meaningful analysis.

Seventy three parents who completed the questionnaire when their child was two years corrected age also completed the same questionnaire when their infant was one year corrected age (data not shown). Four parents gave inconsistent results for current smoking (in two cases in the first year reporting they were ex-smokers and in the second year reporting they were current smokers and in two cases reporting they were current smokers in the first year and never smoked in the second year). Only the second two can really be described as giving ‘incorrect’ inconsistent results.

Table 9.1: Demographic data by viral LRTI status

Data presented as median (range) or n (%).

	RSV LRTI	Other viral LRTI	No LRTI	p
n	13	21	25	
Gestational age (weeks)	33 (23-35)	34 (26-35)	34 (29-35)	0.33
Birth weight (g)	1952 (610-2315)	1826 (670-3154)	2096 (1190-3174)	0.20
Males	6 (46%)	9 (43%)	16 (64%)	0.33
Antenatal smoking	3 (23%)	4 (19%)	2 (8%)	0.41
Antenatal steroids	9 (69%)	16 (76%)	12 (48%)	0.13
Surfactant	2 (23%)	5 (24%)	4 (16%)	0.77
Duration of ventilation (days)	0 (0-81)	0 (0-90)	0 (0-8)	0.82
Bronchopulmonary dysplasia	2 (15%)	3 (14%)	1 (4%)	0.35
Family history of atopy	7 (54%)	14 (67%)	14 (56%)	0.70
Breastfed	11 (85%)	18 (86%)	23 (92%)	0.67
Number of siblings	1 (0-3)	1 (0-5)	1 (0-4)	0.92
Palivizumab in first year	1 (8%)	1 (5%)	0 (0%)	0.33

Table 9.2: Healthcare utilisation by viral LRTI status

Data presented as mean (median) [range].

FIRST YEAR	RSV LRTI (n=13)	Other viral LRTI (n=21)	No LRTI (n=25)	P*
Admission				
Total	1 (0) [0-11]	0 (0) [0-0]	0 (0) [0-1]	<0.0005
Respiratory	1 (0) [0-11]	0 (0) [0-0]	0 (0) [0-0]	<0.0005
A and E				
Total	2 (1) [0-14]	1 (1) [0-4]	1 (0) [0-5]	0.06
Respiratory	2 (1) [0-12]	0 (0) [0-2]	0 (0) [0-3]	<0.0005
Outpatient				
Total	5 (4) [0-14]	5 (3) [0-18]	3 (2) [0-11]	0.21
Respiratory	1 (0) [0-8]	0 (0) [0-9]	0 (0) [0-0]	0.43
GP				
Total	6 (5) [3-12]	6 (5) [1-15]	5 (5) [0-13]	0.50
Respiratory	1 (0) [0-5]	1 (0) [0-4]	0 (0) [0-2]	0.19
SECOND YEAR				
Admission				
Total	0 (0) [0-5]	0 (0) [0-3]	0 (0) [0-1]	0.06
Respiratory	0 (0) [0-3]	0 (0) [0-0]	0 (0) [0-0]	0.004
A and E				
Total	2 (1) [0-14]	1 (0) [0-3]	1 (0) [0-4]	0.39
Respiratory	1 (0) [0-10]	0 (0) [0-1]	0 (0) [0-1]	<0.0005
Outpatient				
Total	1 (0) [0-9]	1 (1) [0-13]	1 (0) [0-8]	0.62
Respiratory	1 (0) [0-6]	0 (0) [0-0]	0 (0) [0-5]	0.21
GP				
Total	5 (3) [1-21]	5 (5) [0-12]	4 (4) [0-14]	0.26
Respiratory	1 (1) [0-6]	1 (1) [0-2]	1 (0) 0-3]	0.16

*The p values relate to the difference between the three groups, post hoc p values are given in the results section text.

Table 9.3: Cost of care (UK£) by viral LRTI status

Data presented as mean [SD]

FIRST YEAR	RSV LRTI (n=13)	Other viral LRTI (n=21)	No LRTI (n=25)	p*
Admission				
Total	3428 [7533]	0 [0]	120 [537]	<0.0005
Respiratory	3350 [7423]	0 [0]	0 [0]	<0.0005
A and E				
Total	248 [489]	77 [101]	52 [98]	0.035
Respiratory	211 [464]	16 [54]	11 [56]	<0.0005
Outpatient				
Total	843 [1237]	869 [1357]	438 [393]	0.29
Respiratory	323 [1165]	244 [1119]	0 [0]	0.43
GP				
Total	296 [269]	340 [260]	228 [190]	0.21
Respiratory	33 [72]	36 [50]	12 [24]	0.19
Medication				
Total	343 [1096]	326 [902]	62 [114]	0.04
Respiratory	259 [927]	201 [906]	2 [5]	0.42
Overall				
Total	4816 [9286]	1285 [1363]	840 [827]	0.038
Respiratory	3917 [8977]	296 [1119]	24 [59]	0.012
SECOND YEAR				
Admission				
Total	824 [2691]	0 [0]	152 [715]	0.06
Respiratory	824 [2691]	0 [0]	0 [0]	0.004
A and E				
Total	240 [454]	56 [88]	57 [84]	0.34
Respiratory	189 [383]	6 [28]	3 [16]	<0.0005
Outpatient				
Total	254 [508]	234 [520]	161 [319]	0.75
Respiratory	88 [283]	0 [0]	34 [132]	0.22
GP				
Total	212 [174]	410 [847]	172 [146]	0.27
Respiratory	62 [79]	27 [27]	24 [38]	0.06
Medication				
Total	64 [118]	231 [796]	25 [42]	0.16
Respiratory	28 [65]	2 [7]	3 [7]	0.005

Overall				
Total	1531 [3649]	700 [1329]	542 [1015]	0.46
Respiratory	1164 [3382]	33 [46]	61 [158]	0.013

*The p values relate to the difference between the three groups, post hoc p values are given in the results section text.

Table 9.4: Respiratory health related questionnaire by viral LRTI status

Data presented as n (%) responding yes to the question.

	RSV LRTI	Other viral LRTI	No LRTI	p*
n	13	21	25	
Did your child cough in the second year?	12 (92%)	20 (95%)	23 (92%)	>0.99
Did your child wheeze in the second year?	6 (46%)	3 (14%)	2 (8%)	0.021
Did your child use any medication for a chest problem in the second year?	6 (46%)	7 (33%)	5 (20%)	0.20
Bronchodilators (e.g. salbutamol)?	3 (23%)	2 (10%)	2 (4%)	0.45
Antibiotics?	6 (46%)	7 (33%)	4 (16%)	0.13
Preventers (e.g. steroids, montelukast)?	1 (8%)	0 (0%)	0 (0%)	0.22
Has your child ever been diagnosed with asthma by a doctor?	2 (15%)	0 (0%)	0 (0%)	0.046

*The p values relate to the difference between the three groups, post hoc p values are given in the results section text.

9.5 Discussion

It has been demonstrated that prematurely born infants developing RSV LRTIs compared to those without LRTIs had significantly higher respiratory health related costs in years one and two. This was associated with an increased respiratory related healthcare utilisation and a greater proportion of the infants who had had a RSV LRTI wheezed and had a doctor's diagnosis of asthma in the second year after birth. Nevertheless, the overall cost of care in year two compared to year one was significantly lower in the RSV group and also in the other two groups.

The greater proportion of the RSV LRTI group having parental reported wheeze and a doctor diagnosis of asthma in the second year than the no LRTI group likely explains their higher costs for respiratory medications. Those results agree with previous studies in children born at term which have demonstrated an increased risk of asthma after RSV LRTI in infancy even in adolescence (Stein et al. 1999; Sigurs et al. 2010).

Only infants in the RSV group required respiratory admissions in either year. In the second year two infants were admitted for RSV LRTIs giving a RSV admission rate of 3.4%. Those results are in keeping with studies reporting admission rates in infancy; the incidence of hospitalisation for RSV infection amongst prematurely born infants has been reported to vary between 2.8% and 37%. In a previous UK study (Thomas et al. 2000) assessing infants born prior to 32 weeks of gestation, a 4% rate was noted. In the PICNIC

study, the overall hospitalisation rate was 3.6% for infants of 33-35 weeks of GA (Wang et al. 1995). In a multicentre Italian birth cohort study (Lanari et al. 2011), 4.5% of infants born between 33 and 37 weeks of gestation were admitted during a six month period for a LRTI, including RSV LRTIs.

In a multicentre randomised trial (1998), prophylaxis with palivizumab was associated with a 55% reduction in RSV hospital admissions, the greatest reduction occurred in the infants who did not have BPD. In this study there was a RSV admission rate of 3.4% which meant that the NNT was 59 assuming a 50% reduction in hospitalisation following prophylaxis. Hence, the cost of RSV prophylaxis would exceed the cost of savings made by a reduction in RSV hospitalisation. A recent double-blind randomised trial (Blanken et al. 2013), however, has demonstrated that palivizumab resulted in a reduction in the total number of wheezing days in the first year after birth. Whether there is a similar impact in the second year after birth requires testing. Nevertheless, given the low costs of health related care in the second year, it still seems unlikely prophylaxis at the current price would be cost effective unless infants at high risk of further admissions with a RSV LRTI could be identified.

To identify high risk infants approaches that could be taken include a clinical decision tree analysis including questions on demographics (e.g. gestational age at birth, birth weight, congenital cardiac disease, neurological impairment, immunodeficiency, maternal smoking, supplementary home oxygen or recently stopped requiring supplementary home oxygen, age at

start of the second RSV season), potential genetic markers (e.g. SNP in ADAM33 although this has only been investigated for infants developing RSV LRTI in the first year after birth) and lung function prior to the start of the second RSV season.

During the course of this thesis, the indications for infants to receive palivizumab changed. At the start of the study palivizumab was given to prematurely born infants with BPD who were being discharged during the RSV season either having very recently come off supplementary oxygen or who would were receiving supplementary home oxygen. By the end of the study the indications for palivizumab changed to a structured approach advised by the JCVI (Joint Committee on Vaccination and Immunisation 2010) including the gestational age at birth and chronological age at the start of the RSV season. Four infants in the cohort received palivizumab, if the subseqeunt guidelines had been followed five infants would have been eligible, i.e. one additional member.

There were no significant differences in healthcare utilisation or health related cost of care between the other viral LRTI group and the no LRTI group in either year. There were also no significant differences between those infants with HRV LRTIs and the no LRTI group, a possible explanation is none of the infants who had a HRV LRTI required hospital admission. Those results suggest that for prophylactic interventions for HRV LRTIs to be cost effective, a high risk group also needs to be identified.

This study had several strengths and some limitations. The prematurely born infants were prospectively followed from birth to two years of corrected age. Symptomatic LRTIs were investigated not only in hospitalised infants, but also in those with LRTIs in the community, which is important, as it has previously been demonstrated that infants with RSV LRTI not requiring hospitalisation also suffer increased respiratory morbidity (Broughton et al. 2005). Healthcare costs in the community as well as those relating to hospital attendances were documented. NPAs were tested by real-time PCR to detect a wide range of respiratory viruses. NPAs were only obtained, however, when the infants were symptomatic and thus it is not possible to comment as to whether asymptomatic LRTIs increase healthcare utilisation at follow-up, but this limitation applies to other studies. The infants included in this study were generally born moderately prematurely, with a median GA of 34 weeks and few required hospitalisation in either years one or two, but they were consecutive infants born at less than 36 weeks of GA who were recruited if parental consent was obtained. Only a proportion of the infants were followed to two years of corrected age, but they did not differ in their demographics to the overall population (data not shown), hence the results are generalisable.

In conclusion, it has been shown that infants developing RSV LRTI in the first year after birth have increased healthcare utilisation and costs in both the first and second year after birth compared to those who had not developed a LRTI. Nevertheless, if RSV prophylaxis in prematurely born infants is to be

cost-effective in the second year after birth a high risk group needs to be identified.

Chapter 10: Discussion

10.1 Summary of the key results

In chapter three, it was reported a single nucleotide polymorphism (SNP) in the ADAM33 gene was associated with the development of RSV LRTIs; this was not associated with a reduction in premorbid lung function. In infants who developed RSV LRTIs, SNPs in genes coding for IL-10, NOS2A and SFTPC were associated with chronic respiratory morbidity and SNPs in genes coding for MMP16, NOS2A, SFTPC and VDR were associated with reduced lung function at one year corrected age, although correction for multiple comparisons was not undertaken.

In chapter four, it was demonstrated that infants with RSV or other viral LRTIs who were hospitalised compared to those who were not had significantly worse premorbid lung function.

In chapter five, it was reported that RSV LRTIs were associated with reduced lung function at one year corrected age, although there were no significant differences in lung function at 36 weeks PMA.

In chapter six, it was reported that the hospitalisation rate of 150 prematurely born infants prospectively followed during the 2009 influenza A (H1N1) pandemic compared to that of term born infants, did not differ significantly for pandemic influenza A (H1N1)v 2009, but was higher for RSV and other viral LRTIs. In addition, it was shown that pandemic influenza A (H1N1)v 2009 immunisation uptake in the prematurely born infants was low (9.3%).

In chapter seven, it was reported that infants developing both HRV and RSV LRTIs had the highest adjusted mean healthcare costs of care, followed by the infants developing HRV LRTIs and then the infants developing RSV LRTIs, compared to infants not developing a LRTI. The HRV group had more out-patient and more respiratory related general practitioner attendances and more wheezed at follow up than the no LRTI group and they had more respiratory related out-patient attendances than the RSV LRTI group.

In chapter eight, it was reported that infants developing HRV-C LRTIs were more likely to wheeze and use respiratory medications and had more days of wheeze and use of an inhaler than the no LRTI group. In addition, the respiratory cost of care was greater for the HRV-C LRTI group than the no LRTI group. Initial analysis demonstrated HRV-A LRTIs were associated apparently with similar effects to HRV-C LRTIs, but the significant differences compared to the no LRTI group disappeared once the infants who also had RSV LRTIs were excluded from the analysis

In chapter nine, it was reported that infants developing RSV LRTIs in the first year have increased healthcare utilisation and costs in both the first and second year after birth compared to those who had not developed a LRTI.

10.2 Relevant research published since commencement of this thesis

10.2.1 RSV

Siezen et al have also demonstrated the same SNP in the ADAM33 gene and a SNP in the IFN-γ gene was associated with an increased risk of hospitalisation with RSV infection in prematurely born infants compared to term born infants (Siezen et al. 2009). Only one other study (Kresfelder et al. 2011) has researched the other SNPs investigated in this thesis, the SNP in the VDR gene was associated with hospitalisation for RSV infection in black South African children (Kresfelder et al. 2011). The differences between that study (Kresfelder et al. 2011) and this thesis may be explained by differences in ethnicity between the populations, maturity at birth and/or the small number of infants with RSV LRTI in this thesis. Several other studies have subsequently identified other SNPs that are associated with RSV infection. SNPs in genes coding for IL-4 and IL-13 (Forton et al. 2009), IL-8 (Lu et al. 2010), surfactant proteins A (El Saleeby et al. 2010) and D (Thomas et al. 2009; Ampuero et al. 2011), IL1RL1 (Faber et al. 2012) and MMP3 (Schuurhof et al. 2012) have been associated with hospitalisation or intensive care admission for RSV infection in infants mainly born at term and from a variety of ethnic backgrounds. One study found no association between a SNP in TLR-4 and RSV infection (Löfgren et al. 2010) and two studies of a SNP in the RANTES gene yielded conflicting results (Tian et al. 2009; Hattori et al. 2011), one suggested the SNP was associated with RSV hospitalisation (Hattori et al. 2011) but the other not (Tian et al. 2009).

This thesis has demonstrated that prematurely born infants who had had RSV LRTIs, a SNP in the gene coding for IL-10 was associated with parental reported wheeze at one year corrected age and SNPs in the genes coding for NOS2A and SFTPC were associated with parental reported cough at one year corrected age. A recent study (Schuurhof et al. 2011) investigated another IL-10 SNP in a cohort of 235 infants hospitalised for RSV bronchiolitis. Although local IL-10 levels during the RSV infection were higher in infants who later developed physician diagnosed post-bronchiolitic wheeze, the IL-10 promoter SNP was not associated with the higher IL-10 concentration in NPAs and no association was observed between the IL-10 SNP and physician diagnosed post-bronchiolitic wheezing. Four other studies have investigated the genetics of post-bronchiolitic wheeze. A RANTES gene SNP was associated with recurrent wheezing (more than one episode of parental reported wheezing) up to three years after hospitalisation for RSV bronchiolitis (Tian et al. 2009). Functional analyses of RANTES promoter activity demonstrated that the RANTES mutation (-403 G to A) increased the transcriptional activity of the RANTES promoter resulting in high serum RANTES levels (Tian et al. 2009). Increased production of RANTES would be predicted to result in enhanced recruitment of proinflammatory cells to the target site, precipitating an amplified local inflammatory reaction (Meurer et al. 1993). Two reports from a cohort of 129 infants hospitalised with bronchiolitis at less than six months of age and followed up at 18 months of age, 68% of whom were RSV positive, highlighted that a SNP in the IFN-γ gene (Nuolivirta et al. 2009) and a mannose binding lectin gene polymorphism were associated with more

severe post-bronchiolitic wheezing requiring treatment with corticosteroids (Nuolivirta et al. 2012). IFN-γ is a proinflammatory cytokine which also seems to have direct antiviral activity (Nuolivirta et al. 2009). Production of IFN-γ is genetically controlled and the allele A at + 874 is associated with low production of IFN-γ. The allele A at + 874 was not only associated with a significantly lower need for corticosteroids used to prevent or treat post-bronchiolitic wheezing but also with protection from multiple respiratory infections (Nuolivirta et al. 2009). Mannose binding lectin (MBL) plays a central role in innate immunity, which is important in early infancy before establishment of adaptive immunity. MBL responds as an acute phase reactant and promotes phagocytosis directly via the lecithin complement pathway. MBL deficiency due to mutations in the MBL gene predisposes to respiratory infections (Nuolivirta et al. 2012). This thesis also found SNPs in genes coding for MMP16 (rs2664349), NOS2A, Surfactant Protein C and VDR were associated with reduced lung function at one year corrected age in infants who had had a RSV LRTI but there have been no other studies on those SNPs.

This thesis has documented reduced lung function (airways resistance measured by whole body plethysmography and resistance of the respiratory system measured by the single breath occlusion technique) at one year corrected age in prematurely born infants who had had a RSV LRTI. A prospective study found that at 18 years adolescents born at term who had been hospitalised for RSV bronchiolitis in infancy had reduced lung function (reduced FEV_1, FEV_1/FVC and FEF_{25-75} z-scores) compared to infants who

had not been hospitalised for RSV bronchiolitis (Sigurs et al. 2010). In contrast, however, a study of monozygotic twins who were discordant for hospitalisation due to RSV infection found no differences in lung function (FEV_1 or sR_{aw}) between the twin pairs at a mean of seven years of age (Poorisrisak et al. 2010). A possible explanation for the lack of significant difference in lung function between the groups was the controls who were the twin siblings of the subject and may have had mild or asymptomatic RSV infection.

This thesis has demonstrated increased healthcare utilisation and associated costs of care in prematurely born infants with RSV and rhinovirus LRTIs compared to those infants who did not develop a viral LRTI. Several recent studies have also demonstrated increased healthcare utilisation and costs in children developing a RSV infection in infancy. Palmer et al (2010) and Stewart et al (2009) retrospectively studied insurance claims data and demonstrated increased healthcare utilisation and associated costs in infants who developed RSV infection, whether it required in-patient or out-patient care compared to matched control infants who did not make an insurance claim for RSV infection. A retrospective study of infants born between 32 and 35 weeks of gestation demonstrated infants hospitalised for RSV infection in infancy had higher healthcare utilisation and associated costs in the first two years after birth compared to those infants who either did not develop a LRTI or were admitted for a non-RSV respiratory cause (Shefali-Patel et al. 2012). Most of the differences in cost in that study were related to the initial hospital admission due to RSV.

10.2.2 Rhinovirus

In this thesis it has been shown that prematurely born infants frequently develop LRTIs caused by rhinovirus (HRV). A study (Miller et al. 2012) of very low birth weight (<1500g), prematurely born infants in Argentina demonstrated 55% of the cohort of 119 infants developed a HRV acute respiratory tract infection. In addition, HRV LRTI accounted for 33% of the 36 infants hospitalised due to viral bronchiolitis, compared to 25% for RSV, and 11% were hospitalised due to dual infections of HRV and RSV (Miller et al. 2012). Infants developing RSV LRTI, however, had more severe disease compared to those with HRV LRTI, as a greater proportion of those developing RSV LRTI required hospitalisation, their hospitalisations were longer and more infants required intensive care and mechanical ventilation (Miller et al. 2012). This thesis has shown that HRV LRTIs, with or without RSV LRTIs, were associated with chronic respiratory morbidity and increased healthcare utilisation and cost of care at follow up and, after excluding infants with RSV infection, HRV-C LRTIs were associated with increased healthcare utilisation and cost of care at follow up.

This thesis demonstrated that in prematurely born infants developing LRTIs caused by viruses other than RSV, reduced premorbid lung function (increased R_{rs}) was associated with hospitalisation for the LRTI. A recent study (van der Zalm et al. 2011) of term born infants, using similar lung function techniques, showed infants who developed wheeze with HRV

infections had higher premorbid R_{rs} than infants who did not wheeze with HRV infections (either upper or lower respiratory tract infections).

HRV LRTI was associated with increased parental reported wheeze at one year corrected age in this thesis. A recent study of term born infants found similar results (Midulla et al. 2012). Multivariate analysis demonstrated hospitalisation with HRV bronchiolitis was an independent risk factor for parental reported recurrent wheeze (more than two episodes of wheeze) one year after initial hospitalisation for HRV bronchiolitis (Midulla et al. 2012). Another recent study demonstrated in term born infants that hospitalisation for HRV bronchiolitis in infancy was associated with wheeze/asthma in adolescence (Ruotsalainen et al. 2012). In addition, the COAST study (Guilbert et al. 2011) showed in high risk (at least one parent with atopy), term born infants, that wheezy HRV but not RSV illnesses in the first three years after birth were associated with reduced lung function (FEV_1, $FEV_{0.5}$ and FEF_{25-75}) at eight years of age. This thesis investigated all viral LRTIs, whether wheeze was a component or not. Infants who had had RSV and HRV LRTIs had the most wheeze and use of anti-asthma medication at follow up at one year of age, followed by those who had HRV LRTIs without RSV LRTIs, suggesting both viruses impact of chronic respiratory morbidity in the first year.

10.2.3 Pandemic Influenza A (H1N1)v 2009

Prematurity was not found to be a risk factor for developing a 2009 pandemic influenza A (H1N1) LRTI requiring hospitalisation, with only one of 140 infants (0.7%) hospitalised. A prospective study of term born infants carried out during the pandemic demonstrated only two infants of 180 (1.1%) developed an influenza A (H1N1) LRTI, one of whom required hospitalisation (Ede et al. 2012). Those data suggest influenza A (H1N1) may have acutely impacted similarly on term and prematurely born infants.

10.3 Strengths of the thesis

10.3.1 Recruitment

The infants in this study were all prematurely born and were recruited while on the newborn unit or maternity ward, that is prior to exposure to viral LRTIs. Despite this, nearly 75% of parents of eligible infants born during the recruitment periods consented for them to be included in the study.

10.3.2 Study population

The infants in this study were all born at less than 36 weeks of gestation and covered a wide range of gestational ages at birth (23 to 35 weeks), although most were of moderate prematurity (median GA of 34 weeks). Prematurely born infants are at high risk of developing RSV and other viral LRTIs and

therefore it is important to assess if they suffer long term respiratory morbidity and to identify risk factors for their chronic respiratory morbidity.

10.3.3 Prospective follow up

Parents of infants were contacted every two weeks; in addition, parents were encouraged to contact the research team whenever their infant was unwell. Nasopharyngeal aspirates were taken from infants either in the community or in hospital when diagnosed with a viral LRTI. Thus, it is likely that most episodes of LRTIs were captured. Although infants developing the most severe viral LRTIs require hospitalisation, the majority of infants developing viral LRTIs are managed in the community. It is therefore important to assess the outcomes of the latter group, which was undertaken in this thesis, as potentially they could be a large healthcare burden. In each results chapter the table of demographics include antenatal smoking as one of the variables. In none was there a significant difference in antenatal smoking between infants who developed viral LRTIs and those who did not. No comparison of maternal smoking within the viral LRTI groups was made between those hospitalised and not hospitalised due to viral LRTIs.

10.3.4 Virus detection

The NPAs were tested for 13 common respiratory viruses by the sensitive technique of real time PCR. There are other viruses, however, including corona viruses and the newly identified WU and KI viruses, which are also

associated with respiratory LRTIs. The impact of those viruses on chronic respiratory morbidity suffered by prematurely born infants also merits investigation.

10.3.5 Lung function assessment at follow up

Lung function assessment at follow up requires sedation. In a previous study (Broughton et al. 2007) 46% of parents gave consent for their infant to undergo sedation and lung function testing at one year corrected age. One hundred and seventy seven infants were recruited and lung function was successfully measured in 78 infants (44%). Lung function was measured at follow up using total body plethysmography, helium gas dilutional functional residual capacity (FRC), compliance and resistance of the respiratory system and FRC and lung clearance index measured by the multiple breath wash-in/out technique. These techniques allowed assessment of lung volumes, as well as small and medium/large airways.

10.3.6 Single nucleotide polymorphism analysis

Infants had their DNA collected at recruitment and then analysed for 11 single nucleotide polymorphisms (SNP) previously related to RSV infection, prematurity or BPD.

10.4 Weaknesses of the thesis

10.4.1 Hospitalisations

Infants were assessed both in the community and in hospital, but only ten infants who developed RSV LRTIs were hospitalised due to the LRTI (34.5% of all infants developing RSV LRTIs and 6.5% of the whole cohort) and only two infants who developed other viral LRTIs were hospitalised due to the LRTI (6.5% of all infants developing other viral LRTIs and 1.3% of the whole cohort). The results relating to the data at follow up, therefore, are more applicable to infants with viral LRTIs managed in the community. In addition, all the infants were symptomatic when assessed and thus the impact of asymptomatic viral infections could not be assessed in this thesis.

10.4.2 Gestational age

Although infants in the range 23 to 35 weeks of gestation at birth were recruited in this study, their median GA at birth was 34 weeks. The results of this study may not, therefore, be representative of the most prematurely born infants.

10.4.3 Sample size

A SNP in the ADAM33 gene was associated with an increased risk of developing RSV LRTIs and SNPs in several other genes were associated

with increased chronic respiratory morbidity and reduced lung function at one year corrected age in infants who had had RSV LRTIs. The genetic analysis, however, was not powered to correct for multiple testing so it is possible certain of the results could be due to chance. In addition, amongst the infants with RSV LRTIs, although there were no significant differences in ethnic origin for each of the 11 SNPs, the numbers in each group do not allow a clinically meaningful conclusion to be drawn.

10.4.4 Viral testing

NPAs were tested for 13 viruses using real-time PCR. Other viruses causing respiratory tract infections in infants (e.g. coronaviruses and newly identified polyomaviruses) were not investigated in this thesis and thus infants with viral negative LRTIs may have been infected with a virus not investigated in this thesis. In addition, viral shedding from an infant's respiratory system can occur for many weeks after recovery from an acute illness (Peltola et al. 2013). Thus it is not possible to be certain that any virus detected was necessarily the pathogen causing the infant's clinical symptoms at the time of the illness.

Although infants were closely followed, regular NPAs were not undertaken when infants were asymptomatic. It was, therefore, not possible to establish the relationship between exactly when an infant was infected with a virus and the development of clinical symptoms. This is a limitation in many previous

studies (Lemanske et al. 2005; Broughton et al. 2007; Janssen et al. 2007; Guilbert et al. 2011; Ermers et al. 2011).

In Chapter four, 13 (22%) of the 58 infants who developed viral LRTIs with a virus detected and 8% of all the 159 infants followed over the first RSV season had more than one virus detected from one NPA. Previous studies have demonstrated rates of multiple virus detection between 0-70% depending on the population studied and the techniques used to test respiratory samples (Lazar et al. 2004, Konig et al. 2004, Semple et al 2005, van Woensel et al. 2006, Richard et al. 2008, Harada et al. 2013).

10.4.5 Lung function testing by the multiple breath wash-in/out technique

Lung function was undertaken using the multiple breath wash-in/out technique at both 36 weeks and one year corrected age. At 36 weeks corrected age the infants had very irregular breathing patterns which meant obtaining high quality data was extremely difficult. The co-efficient of variation (CoV) at 36 weeks corrected age was 16% for FRC_{MBW} and 13% for LCI. When undertaken at one year corrected age, in sedated infants with regular respiration, the technique produced much more reliable data (FRC_{MBW} CoV 8% and LCI CoV 6%).

10.4.6 Comparison with a term born control group

This study only recruited prematurely born infants. Recruitment of term born infants would have allowed comparison of risk factors for developing viral LRTIs between term and prematurely born infants, but would have required a much longer study and would not have addressed the hypotheses of this thesis.

10.5 Clinical implication of the results

Palivizumab, a monoclonal antibody used as immunoprophylaxis against RSV, is available for use in prematurely born infants. Due to the high cost, however, palivizumab is only available to those prematurely born infants deemed to be at the highest risk, currently based on GA and chronological age at the start of the RSV season (Joint Committee on Vaccination and Immunisation 2010). These criteria may miss some infants who are at higher risk of developing RSV infection based on other risk factors and include some infants at lower risk. Premorbid lung function and genotyping may further identify high risk infants.

In a non-randomised trial palivizumab was shown to reduce recurrent wheeze in prematurely born infants (Simoes et al. 2007) and in a randomised controlled trial to reduce days of wheeze in the first year of life (Blanken et al. 2013). Results from this thesis have shown that RSV and HRV LRTIs are associated with chronic respiratory morbidity and associated healthcare utilisation and associated cost of care in prematurely born infants. Those results highlight that development of therapies directed against HRV infection

might further reduce the chronic respiratory morbidity suffered by prematurely born infants.

A low uptake of the influenza A H1N1 immunisation in prematurely born infants during the pandemic in 2009 was demonstrated highlighting that infants need to be better targeted with immunisation campaigns to ensure as many infants as possible receive their immunisations.

10.6 Future studies

Studies are required to determine whether an algorithm including more risk factors, including results from this thesis might better identify high risk infants requiring prophylaxis in either the first or second years after birth.

There is currently no prophylactic agent for rhinovirus infection but more studies are required to assess the long term respiratory impact of this and other respiratory viruses on term and prematurely born infants.

A larger study, adequately powered for multiple comparisons, is required to confirm the findings from the genetics component of this thesis and to assess the influence of ethnicity on genetic predisposition to RSV LRTI. Studies investigating genetic markers which may predispose infants to other viral LRTIs are also required. Increased knowledge of genetic markers would allow improved targeting of prophlyaxis and education of parents to reduce

the risk of developing viral LRTIs. It may also lead to the development of novel treatments or prophylactic agents for viral LRTIs.

10.7 Conclusion

Respiratory viral LRTIs are associated with increased chronic respiratory morbidity at follow up. Certain infants have a functional and genetic predisposition to developing severe RSV infections.

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Appendices

Appendix 1- An example of a diary card

JULY 2009

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
Notes: For each day please mark if your child: Coughed (C) Wheezed (W) Took any medicine for a chest problem e.g. inhaler, antibiotic (M) Visited your GP or A&E because they were unwell (rather than a follow up appointment) (H)			1	2	3	4
5	6	7	8	9	10	11
12	13	14	15	16	17	18
19	20	21	22	23	24	25
26	27	28	29	30	31	

Appendix 2- Respiratory health related questionnaire

Diminished lung function, viral infections and chronic respiratory morbidity in prematurely born infants

Study Number: 08/H0808/37

Thank you for taking the time to fill out this questionnaire. Please circle the most appropriate answer for your child or fill in the box. If there are any questions you do not understand or have any queries, please phone Dr Simon Drysdale or Mireia Alcazar on 020 3299 8494/07766 759399 or Consultant Paediatrician Anne Greenough on 020 7346 3037.

Patient Identification Number:…….

Baby's full name. . . . . .

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1. Chest Symptoms

1.1 Did your child cough in the first 12 months of life?……..……..Yes/No

If yes indicate how often:

More than once a week……Yes/No
Once a week or less but more than once a month…….…..Yes/No
Once a month or less……..…Yes/No

If yes, was the cough brought on by:

Exercise/Activity…….Yes/No/Don't Know
Infection/a cold……Yes/No/Don't Know

1.2 Did your child wheeze in the first 12 months of life?……...……Yes/No

If yes indicate how often:

More than once a week…….Yes/No
Once a week or less, but more than once a month……..Yes/No
Once a month or less…….Yes/No

In the first 12 months, was the wheeze brought on by:

Exercise/Activity…….Yes/No/Don't Know
Infection/a cold……...Yes/No/Don't Know

1.3 Has your child ever required oxygen (via mask or nasal prongs) at home?…....Yes/No

If yes:

Does he/she still require oxygen…….Yes/No

At what age did he/she stop requiring home oxygen? ☐

Treatment for chest symptoms

1.4 Has your child had medicines for chest problems?……..…Yes/No

If yes indicate

Bronchodilators (relievers) e.g. ventolin/bricanyl/atrovent/serovent…..Yes/No
Antibiotics……...Yes/No
Preventers e.g. Becotide/Pulmicort/Flixotide/steroids by mouth…….Yes/No
Any other (please name) ……..

1.5 Has your child received treatment (Synagis/Palivizumab) to try and prevent them getting RSV infection (bronchiolitis)?…….Yes/No

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2. Hospital Admissions

2.1 Has your child ever been admitted to hospital because of breathing difficulties?……..…Yes/No

If yes:

Age of child	Diagnosis	Hospital	Approx stay

2.2 Has your child ever been admitted to hospital with bronchiolitis?…….Yes/No

If yes:

Age of child	Diagnosis	Hospital	Approx stay

2.3 Has he/she ever been admitted to hospital for any other reason?……..Yes/No

If yes:

Age of child	Diagnosis	Hospital	Approx stay

2.4 Has he/she ever been admitted to a children's intensive care unit?……...Yes/No

If yes

Age of child	Diagnosis	Hospital	Approx stay

3. Other Questions

3.1 Has your child been diagnosed with:

Asthma ……Yes/No

Eczema……Yes/No

3.2 Has anyone in the child's immediate family (e.g. the child's mother, father, or brother/sisters) been diagnosed with:

Asthma ……Yes/No
Eczema……Yes/No
Hayfever……Yes/No

3.3 Did you breast feed your child?……..…Yes/No

If yes for approximately how long? ☐

3.4 Have you smoked since having your baby?

Never …… ☐
Ex - i.e. stopped before/during pregnancy - not resumed.. ☐
Current …… ☐

If current, how many per day:

	Social/occasional (less than one per day)
	1 – 10 per day
	11 – 20 per day
	21 – 30 per day
	31 or more per day

3.5 Does your partner (or other household members) smoke?……Yes/No

Does your child have contact (more than 15 hours per week) with anyone who smokes?…….……..…Yes/No

3.6 How many children under the age of five **(*including the child in this study*)** are living at your house? ☐

3.7 Do you have pets living in the house?……..Yes/No

If yes please specify

☐

3.8 What best describes your type of accommodation?

Owns it outright…….Yes/No
Owns with mortgage or loan……Yes/No
Pays part rent, part mortgage……Yes/No
Rents from council or housing association……Yes/No
Rents from Private Landlord……Yes/No
Live there rent free……Yes/No
Other (please state)…….

3.9 Please use the space below to tell us anything else you think may be useful

……

……

……

……

……

……

If you are unsure about anything regarding this form, please do not hesitate to contact Dr Simon Drysdale or Mireia Alcazar on 020 3299 8494/07766 759399.

Thank you for taking the time to fill out this form.

Appendix 3- Patient information sheet and consent form

Patient Information Sheet Version 2, April 2008

Diminished lung function, viral infections and chronic respiratory morbidity in prematurely born infants

We are inviting you to consider allowing your baby to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with your friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish your baby to take part.

Why are we doing it?

Infants who are born prematurely frequently have breathing problems at follow up, wheeze and cough requiring treatment. It is possible that viral infections may make those problems worse. In infants born at the correct time (at term), however, there is evidence to suggest that viral infections and subsequent wheezing occur in those with prior abnormal lung function and/or a genetic predisposition and thus the viral infection itself may have no role in increasing breathing problems at follow up. There is no similar information for prematurely born infants. Yet, in those babies it is particularly important to know if viral infections do play a role in the breathing problems and whether certain babies are more susceptible, as there is treatment available which can reduce the severity of the acute viral infections. It is, however, very expensive and has to be given by five monthly injections so it is important only to give the treatment to babies who would really benefit from it. Hence, the aim of our study is to investigate whether abnormal lung function prior to neonatal unit/postnatal ward discharge predisposes prematurely born babies to respiratory viral infections and if viral infections contribute to the breathing problems at follow up suffered by prematurely born infants. In addition, we will determine if some infants have a genetic predisposition to viral infections and wheezing.

How we will do it?

We wish to assess the lung function of prematurely born babies just prior to them being discharged from the neonatal unit or postnatal ward. To assess lung function we will use simple breathing tests which are used routinely in clinical practise to assess babies with breathing problems. The baby breathes in and out through a facemask which is attached to a device which measures the amount of air the baby is breathing in and out and how much effort the baby is putting into breathing. The facemask is then connected to another device which contains a small amount of an inert (non harmful) gas. Each test takes less than five minutes and from the data collected we can assess the size of the baby's lungs and any difficulties of breathing in and out. If the baby is having a blood test for clinical purposes then we would wish to collect an extra one millilitre (less than half a teaspoonful of blood). If not we would like to collect a buccal smear from your baby and this requires lightly brushing the inside of the cheek with a swab. The blood and buccal smears will be analysed for the variations in genes which have been shown to be associated with viral infections and subsequent wheezing in babies born at term. We also wish to take blood samples from you as, because of the size of the samples that can be taken, samples from adults are easier to analyse. It is, however, important to also have samples from your baby as sometimes variations in genes occur spontaneously in babies and are not inherited from their parents.

We then wish to follow the babies until they are one year of age corrected for the degree of prematurity at birth. From October to March, we will ask parents to contact us every time their baby has symptoms which could be due to a viral infection, at home or hospital. The research doctor or nurse would then arrange to visit your baby and a nasopharyngeal aspirate would be taken. A nasopharyngeal aspirate is obtained by using a small "swab" on a stick to collect secretions from just inside the baby's nose. The secretions are then analysed to determine if the baby had a viral infection and which viral infection. When your baby is 11 months corrected age we will ask you to complete a diary card for one month, that is to record on a daily basis whether baby coughed, wheezed or required treatment. For babies recruited early in the study we will also ask parents to complete a second diary card when their child is nearly two years of age.

When your baby is one year of age, we wish to again assess your baby using the same breathing tests as before, but additionally assess the size of the breathing tubes (which may be reduced in babies with troublesome wheeze) by assessing the baby in a plethysmograph, which is similar to an incubator. Again the baby breathes in and out through a facemask. All breathing tests at one year of age require the baby to be sedated. The sedation is given orally, is very safe and is used routinely in clinical practise to assess babies with actual or suspected breathing problems. Throughout the breathing tests, the baby is continuously monitored and looked after by a paediatrician.

We would also like to assess how much treatment your baby has required over the first year and to do this we wish to examine the medical notes held by the GP and hospital. From those notes we will be able to determine the number of times the baby has been to hospital, how long was spent there, plus the type of treatment the baby received in hospital and from their GP.

We will compare all of the results between babies who have had viral infections and those who have not and hence determine whether viral infections do cause breathing problems at follow up and if certain babies are particularly at risk.

Travel expenses

Reimbursement for appropriately incurred travel expenses is available on request.

Are there any benefits or disadvantages to take part in this study?

The benefits of taking part are that your baby will be closely followed and any breathing problems will be detected early and receive treatment. The diagnosis of a viral infection when the baby is acutely unwell will aid the choice of treatment your baby will receive. There are no risks to your baby taking part in this study, all of the tests are those used in routine clinical practise. There are no disadvantages, but no benefits to your baby regarding the assessment of genetic predisposition. As it is not known whether prematurely born infants do have a genetic predisposition to viral infections, we will only analyse the samples at the end of the study when all the information is available. The results, however, will provide essential information for the care of future babies, that is whether certain babies are genetically predisposed to viral infections and wheeze at follow up and thus may benefit from preventative treatment.

How will the information be shared?

All information that is collected will be kept strictly confidential. Results form the study will be published in scientific journals in which all data are anonymous. We will, with your consent, inform your GP of your child's involvement in this study.

Giving consent

It is important you understand that this study is voluntary and for research purposes. You may choose not to allow your baby to participate in this study and you may withdraw your child at any time without their medical care or legal rights being affected. If you require any further information please ask. There are no harmful side effects associated with this study. No indemnity is available, although normal NHS procedures apply in the unlikely event of any adverse outcome of the study.

If you decide to allow your baby to take part in the study you will be given a copy of the information sheet and consent form to keep. All information that is collected during the course of the research will be kept strictly confidential and if the results are published then anonymity will be maintained. This study was reviewed and approved by the Research Ethics Committee of King's College Hospital NHS Foundation Trust.

Thank you very much for considering your baby for enrolment into this project. If you are happy to participate please sign below. If you have any further questions or would like to meet with a member of the research team to discuss this further, please do not hesitate to contact us on the telephone number below.

Dr Simon Drysdale
Paediatric Clinical Research Fellow
Tel: 020 3299 8494
Mob: 07766 759399

Professor Anne Greenough
Professor of Neonatology and
Clinical Respiratory Physiology

Direct line: 020 3299 3037

Centre Number: KCH
Study Number: 08/H0808/37

Patient Identification Number for this trial:

CONSENT FORM

Title or project: **Diminished lung function, viral infections and chronic respiratory morbidity in prematurely born infants**

Name or researcher: Professor Anne Greenough

Please initial box

1. I confirm that I have read and understand the information sheet Dated April 2008 (version 2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my child's participation is voluntary and that my child is free to withdraw at any time, without giving any reason, without his/her medical care or legal rights being effected. ☐

3. I understand that relevant sections of any of my child's medical notes and data collected during the study, may be looked at by responsible individuals from King's College London, from regulatory authorities or from the NHS Trust, where it is relevant to my child taking part in this research. I give permission for these individuals to have access to my child's records. ☐

4. I agree to my child's GP receiving copies of tests results from this study ☐

5. I agree for my child to take part in the above study. ☐

| --- | --- | --- |
Name or parent/Guardian	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

When completed, 1 for patient, 1 for researcher site file; 1 (original) to be kept in medical notes

Appendix 4- Ethical approval confirmation letter

King's College Hospital Research Ethics Committee
Camberwell Building
King's College Hospital
94 Denmark Hill
London
SE5 9RS

Telephone: 0203 299 3923
Facsimile: 0203 299 5085

17 April 2008

Prof Anne Greenough
Professor of Neonatology and Clinical Respiratory Physiology
Guy's, King's & St Thomas' School of Medicine
Frederick Still Ward, 4th Floor Golden Jubilee Wing,
King's College Hospital, Denmark Hill, London
SE5 9RS

Dear Prof Greenough

Full title of study: **Diminished lung function, viral infections and chronic respiratory morbidity in prematurely born infants**
REC reference number: **08/H0808/37**

Thank you for your letter of 7th April 2008, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the sites outlined on the site approval form. We will write to you again as soon as any further Research Ethics Committee has notified the outcome of a SSA.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*
Application	26/03/2008
Investigator CV	Anne Greenough
Protocol	
GP/Consultant Information Sheets	Dated 18th February 2008
Participant Information Sheet	Version 2 April 2008
Participant Consent Form	Version 2 April 2008
Response to Request for Further Information	Dated 7th April 2008

Please note that your Patient Information Sheet and Consent form have been issued with Version numbers as none were supplied. Please ensure that this are entered onto the documents before they are used.

R&D approval

All researchers and research collaborators who will be participating in the research at NHS sites should apply for R&D approval from the relevant care organisation, if they have not yet done so. R&D approval is required, whether or not the study is exempt from SSA. You should advise researchers and local collaborators accordingly.

Guidance on applying for R&D approval is available from http://www.rdforum.nhs.uk/rdform.htm.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

Here you will find links to the following

a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service on the application procedure. If you wish to make your views known please use the feedback form available on the website.
b) Progress Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
c) Safety Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
d) Amendments. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
e) End of Study/Project. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nationalres.org.uk .

08/H0808/37	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Dr David Jewitt
Chair
Email: william.bowen@kch.nhs.uk

Enclosures: *Standard approval conditions*
Site approval form

Copy to: Dr Lorraine Catt

King's College Hospital Research Ethics Committee
LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	08/H0808/37	**Issue number:**	0	**Date of issue:**	17 April 2008
Chief Investigator:	Prof Anne Greenough				
Full title of study:	Diminished lung function, viral infections and chronic respiratory morbidity in prematurely born infants				

This study was given a favourable ethical opinion by King's College Hospital Research Ethics Committee on 30 April 2008. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes* (1)
Prof. Anne Greenough	Professor of Neonatology	*King's College Hospital NHS Foundation Trust*	*King's College Hospital Research Ethics Committee*	*17th April 2008*	

Approved by the Chair on behalf of the REC:

…… *(Signature of Co-ordinator)*

……. (Name)

(1) *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded*

Appendix 5- Overall flow diagram of infants in the study

Doesn't include Chapter 6